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(54) Title: NOVEL LIPOLYTIC ENZYMES

(57) Abstract

Certain specified variants of a parent lipase belonging to the *Pseudomonas* sp. lipase family have improved properties, in particular improved washing properties. The lipase variants of the invention have an amino acid sequence comprising substitution, deletion or insertion of an amino acid at one or more specified positions of the parent lipase. The invention also provides methods of preparing such lipase variants.

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NOVEL LIPOLYTIC ENZYMES

FIELD OF THE INVENTION

The present invention relates to modified lipolytic enzymes of particular interest for use in detergent or cleaning compositions.

BACKGROUND OF THE INVENTION

For a number of years lipolytic enzymes have been used as detergent enzymes, *i.e.* to remove lipid or fatty stains from clothes and other textiles. For instance, various microbial lipases have been suggested as detergent enzymes. Examples include lipases derived from *Humicola lanuginosa*, e.g. described in EP 258 068 and EP 305 216, from *Absidia* sp. (WO 96/13578), a *Pseudomonas* lipase such as a *Ps. alcaligenes* and *Ps. pseudoalcaligenes* lipase, e.g. as described in EP 218 272, *Ps. cepacia*, e.g. as described in EP 331 376, *Pseudomonas sp.* as disclosed in WO 95/14783, *Ps. mendocina* (also termed *Ps. putida*), *Ps. syringae*, *Ps. aeruginosa, Ps. wisconsinensis* (WO 96/12012) or *Ps. fragi* (EP 318 775), *from Bacillus*, e.g. *B. subtilis* (Dartois et al., 1993), *B. stearothermophilus* (JP 64/744992) and *B. pumilus* (EP 91 00664).

Other types of lipolytic enzymes having been suggested as detergent enzymes include so-called cutinases, e.g. derived from *Pseudomonas mendocina* as described in WO 88/09367, or from *Fusarium solani pisi* (e.g. described in WO 90/09446).

In recent years attempts have been made to prepare lipase variants having improved properties for detergent purposes. For instance, WO 92/05249 discloses lipase variants with improved properties, in which certain characteristics of wild-type lipase enzymes have been changed by specific, i.e. site-directed modifications of their amino acid sequences. More specifically, lipase variants are described, in which one or more amino acid residues of the so-called lipid contact zone of the parent lipase has been modified.

WO 94/01541 describes lipase variants with improved properties, in which an amino acid residue occupying a critical position vis-a-vis the active site of the lipase has been modified.

EP 407 225 discloses lipase variants with improved resistance towards proteolytic enzymes, which have been prepared by specifically defined amino acid modifications.

EP 260 105 describes hydrolases in which an amino acid residue within 15 Å from the active site has been substituted.

WO 95/35381 discloses *Pseudomonas* sp. lipase variants, in particular *P. glumae* and *P. pseudoalcaligenes* lipase variants which have been modified so as to increase the hydrophobicity at the surface of the enzyme.

WO 96/00292 discloses *Pseudomonas* sp. lipase variants, in particular *P. glumae* and *P. pseudoalcaligenes* lipase variants which have been modified so as to improve the enzyme's compatibility to anionic surfactants,

WO 95/30744 discloses mutant lipases such as *Pseudomonas* sp. lipases which have been modified to an increased surfactant resistance

WO 94/25578 discloses mutant lipases comprising at least a substitution of the methionine corresponding to position 21 in the *P. pseudoalcaligenes* lipase, in particular to leucine, serine or alanine.

WO 95/14783 discloses mutants of the *Ps. mendocina* lipase SD702.

WO 95/22615 discloses variants of lipolytic enzymes having an improved washing performance, the variants having been prepared by a method involving subjecting a DNA sequence encoding the parent lipolytic enzyme to random mutagenesis and screening for variants having a decreased dependence to calcium and/or an improved tolerance towards a detergent or one or more detergent components as compared to the parent lipolytic enzyme.

WO 95/09909 discloses, inter alia, chemically modified lipases or lipase mutants which has a higher pl than the corresponding modified enzyme.

As it will be apparent from the above disclosure *Pseudomonas* sp. lipases constitute one group of lipases of interest as detergent lipases. In particular, the *Ps. pseudoalcaligenes* (also termed *Ps. alcaligenes)* lipase has been mentioned to be of interest for detergent purposes. On the basis of amino acid sequence and/or structural homology a number of different *Pseudomonas* lipases are considered to belong to the same family of lipases. More specifically, the lipases from the following sources have been found to have a high degree of amino acid sequence homology, such as at least 40 %, at least 60% homology, at least 80% homology or at least 90% homology, and thus are contemplated to belong to the same family of lipases: *Ps. sp.* ATCC21808, *Pseudomonas* sp. lipase commercially available as Liposam®, *Ps. mendocina* SD702, Ps. *aeruginosa* EF2, *Ps. aeruginosa* PAC1R, *Ps. aeruginosa* PAO1, *Ps. aeruginosa* TE3285, *Ps. sp.* 109, *Ps. pseudoalcaligenes* M1, *Ps. glumae*, *Ps. cepacia* DSM3959, *Ps. cepacia* M-12-33, *Ps. sp.* KWI-56, *Ps. fragi* IFO3458, *Ps. fragi* IFO12049 (Gilbert, E. J., (1993), Pseudomonas lipases: Biochemical properties and molecular cloning. Enzyme Microb. Technol., 15, 634-645). The species *Pseudomonas cepacia* has recently been reclassified as *Burkholderia cepacia*, but is termed *Ps. cepacia* in the present application.

There is an ever existing need for providing novel lipases with improved properties, in particular improved washing properties. The present invention relates to such novel lipases.

SUMMARY OF THE INVENTION

In its broadest aspects, the present invention relates to certain specified variants of a parent lipase, which belongs to the *Pseudomonas* sp. lipase family and to a method of constructing such variants. More specifically, in its first aspect the invention relates to a variant of a parent lipase which has been modified at one or more positions corresponding to the following positions of mature, wild-type *Ps. pseudoalcaligenes* lipase having the amino acid sequence specified in EP 334 462:

a) substitution of an amino acid residue at a position corresponding to one of the following positions in the mature, wild-type *Ps. pseudoalcaligenes* lipase: G1, L2, F3, G4, S5, T6, G7, K12, I15, T18, M21, L22, D25, S26, I27, L28, D31, W33, Y34, G35, S38, S39, R41, S42, D43, S46, Y48, I49, T50, E51, S53, Q54, L55, N56, T57, E59, L60, E63, E64, L66, E67, V69, E70, E71, I72, A73, I75, S76, K78, G79, V84, H88, G90, V93, Y95, V96, A98, V99, D102, V110, G111, A112, P113, H114, D118, T119, D121, F122, I123, Q125, I126, P128, A131, G132, E133, A134, I135, V136, A137, G138, V140, G142, L146, F149, S153, S155, T156, S157, A161, L162, G163, A164, E166, S167, N169, S170, E171, A173, A174, A175, F176, A178, K179, Y180, Q182, I184, P185, A188, G190, E191, G192, A193, Y194, K195, V196, N197, G198, V199, S200, S205, T207, S208, P209, L210, D215, V216, S217, D218, L219, L220, G222, A223, S224, S225, T227, D229, E230, P231, D233, R238, S241, H242, K245, R248, D249, D250, R252, L256, D257, E258, V259, Q261, T262, L265, T266, S267, L268, F269, E270, D272, T275, V276, Q280, L286, A287, and L289; and/or

b) insertion of an amino acid residue between the amino acid residues located in a position of the parent lipase corresponding to positions G29 and V30; to positions I126 and P127; to positions A188 and C189; to positions A193 and Y194; to positions D229 and E230; and/or positions E230 and P231 of the wild type *Ps. pseudoalcaligenes* lipase; and/or

c) deletion of an amino acid residue at a position corresponding to one of the following positions in the mature, wild-type *Ps. pseudoalcaligenes* lipase: G1, L2, F3, G4, S5, T6, G7, I75, S155, T156, S157, L265, T266, S267, L268, F269, E270.

In the present context the term "*Pseudomonas* sp. lipase family" is intended to indicate a family of lipases which show a high degree of homology on the amino acid or the structural level. More specifically, the term is intended to indicate lipases which show at least 40 % homology, e.g. at least 60% homology, such as at least 80% homology or at least 90% homology. The polypeptide homology is determined as the degree of identity between the two sequences indicating a derivation of the first sequence from the sec-

ond. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453. Using GAP with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

Specific examples of lipases belonging to the *Pseudomonas* sp. lipase family may be isolated from the following organisms or may be the following: *Ps. sp.* ATCC21808, *Pseudomonas* sp. lipase commercially available as Liposam®, Ps. *aeruginosa* EF2, *Ps. aeruginosa* PAC1R, *Ps. aeruginosa* PAO1, *Ps. aeruginosa* TE3285, *Ps. sp.* 109, *Ps. pseudoalcaligenes* M1, *Ps. glumae*, *Ps. cepacia* DSM3959, *Ps. cepacia* M-12-33, *Ps. sp.* KWI-56, *Ps. fragi* IFO3458, *Ps. fragi* IFO12049 (Gilbert, E. J., (1993), Pseudomonas lipases: Biochemical properties and molecular cloning. Enzyme Microb. Technol., 15, 634-645), *Ps. fragi*. The species *Pseudomonas cepacia* has recently been reclassified as *Burkholderia cepacia*, but is termed *Ps. cepacia* in the present application.

The position corresponding to a given position in the *Ps. pseudoalcaligenes* lipase may be determined by alignment of the sequence of the parent enzyme in question with that of the *Ps. pseudoalcaligenes* lipase, e.g. using the alignment disclosed by Svendsen et al, Biochimica et Biophysica Acta, 1259 (1995) 9-17. Alternatively or in addition, the corresponding position may be identified by superimposing the three-dimensional structure of the relevant lipases.

In a second important aspect the invention relates to a method of constructing a variant lipase from a parent lipase belonging to the *Pseudomonas* sp. lipase family, which variant has an improved wash performance as compared to the parent lipase, which method comprises:

subjecting an amino acid subsequence region of the parent lipase which corresponds to one of the following amino acid subsequences 6-14, 15-18, 21-36, 37-39, 46-74, E59-V84, 88-100, 109, 110-136, D118-A145, F149-A173, 154-167, 168-180, A174-S205, 188-200, G206-C239, 222-231, S240-P273, 258-266 or 267-268 of the mature, wild-type *Ps. pseudoalcaligenes* lipase to localized random mutagenesis,

expressing the variety of mutated DNA sequences originating from the parent lipase obtained in step (a) in suitable host cells; and

screening for host cells expressing a mutated lipase which has a decreased dependence on calcium and/or an improved tolerance towards a detergent or a detergent component as compared to the parent lipase.

In the present context, the term "localized random mutagenesis" is intended to indicate random mutagenesis being conducted of a specific limited part of the parent enzyme.

The term "random mutagenesis" is intended to be understood in a conventional manner, i.e. to indicate an introduction of one or more mutations at random positions of the parent enzyme or introduction of random amino acid residues in selected positions or regions of the parent enzyme. The random mutagenesis is normally accompanied by a screening which allows the selection of mutated lipolytic enzymes which, as compared with the parent enzyme, have improved properties. Suitable techniques for introducing random mutations and screening for improved properties are described in WO 95/22615.

The term "improved wash performance" as used about lipase variants disclosed herein is intended to indicate that the enzyme has an improved performance when tested in a suitable wash assay or a wash related assay (such as the assays described in the Materials and Methods section herein) as compared to the parent enzyme. The improved performance may be in terms of lipid stain removing capability and/or a decreased calcium dependency, an improved tolerance towards a detergent or detergent component, an increased hydrophobicity, an interesting substrate specificity, an improved one-cycle wash effect, etc.

In the present context, the term "decreased dependence on calcium" as used in connection with the screening for mutated lipases, in particular lipases exhibiting enzymatic activity towards lipase substrates having hydrocarbon chains (ffa-part) of a length exceeding approx. 6-8 C-atoms, is intended to mean that the mutated lipase requires lower amounts of Ca^{2+} for exhibiting the same degree of activity and/or stability as the parent enzyme when tested under similar conditions. In other words the stability and/or activity of the mutated enzyme is/are increased in the absence of calcium as compared to that of the parent enzyme. The stability may, e.g., be assayed by a determination of residual activity upon preincubation under Ca-free conditions and/or DSC (Differential Scanning Calorimetry) in the absence/presence of free Ca2+. Preferably, the mutated lipase of the invention is substantially independent of the presence of calcium for exhibiting enzymatic activity, in particular at a pH higher than 8.

The term "improved tolerance towards a detergent or detergent component" as used in connection with the screening for mutated lipases is intended to mean that the mutated lipase is active at higher concentrations of the detergent or detergent component than the parent enzyme.

In the present context the term "detergent" is intended to indicate a mixture of detergent ingredients normally used for washing or dishwashing. Analogously, a "detergent component" is intended to indicate a component or ingredient normally found in detergent or dishwashing compositions, specific examples of which are given in the section further below entitled "Detergent compositions".

Further, the invention provides a method of preparing a variant lipase, comprising:

a) selecting a first and a second *Pseudomonas* lipase having amino acid sequences which can be aligned so that more than 40 % of the amino acid residues are identical,

b) identifying substitutions, insertions and deletions whereby the second sequence differ from the first sequence,

c) designing a variant amino acid sequence by introducing one or more of said substitutions, insertions and deletions into the first amino acid sequence,

d) designing a DNA sequence expressing said variant amino acid sequence,

e) preparing said DNA sequence and transforming a suitable host organism by inserting the DNA sequence,

f) cultivating the transformed host organism to express and secrete a lipase having the variant amino acid sequence and recovering the lipase.

In the context of the present invention, the degree of amino acid sequence homology is determined as the degree of identity between two sequences when optimally aligned, indicating a derivation of the second sequence from the first. The homology may suitably be determined by means of computer programs known in the art, such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711), (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453).

In further aspects, the invention relates to a DNA construct encoding a lipase variant of the invention, an expression vector harboring the DNA construct, a host cell comprising the DNA construct, optionally present on a vector, which cell may be used for the recombinant production of the lipase variant. Finally, the invention relates to a detergent composition comprising a lipase variant of the invention.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 shows a restriction map of pYellow002.

Fig. 2 shows a restriction map of pNOVO Lip/Lim.

Fig. 3 shows a restriction map of the synthetic gene shown in SEQ ID NO: 1.

Fig. 4 shows a restriction map of pNovoNordisk-PSD.

Fig. 5 shows a restriction map of the synthetic gene shown in SEQ ID NO: 2.

Fig. 6 shows a restriction map of pWYLM.

FIG. 7 shows a construction of pUC119SDL195+SDL451 and pMES6.

Fig. 8 shows the construction of BEN2EBpBSII and pHSG397ppBEN2.

Fig. 9 shows the construction of pTRBEN2.

Fig. 10 shows examples of the construction of plasmids containing genes encoding module shifted *Ps. pseudoalcaligenes* lipase variants in BEN2EBpBSII, pHSG397ppBEN2, or LMNBpUC. Fig.10 also shows the construction of LMNBpUC.

Fig.11 and 12 show the construction of genes encoding module shifted *Ps. pseudoalcaligenes* lipase variants.

Fig.13 shows examples of the construction of plasmids containing genes encoding module shifted *Ps. pseudoalcaligenes* lipase variants in pMES6 or pMLM3R. Fig.13 also shows the construction of pMLM3R.

DETAILED DESCRIPTION OF THE INVENTION

In one embodiment the lipase variant of the invention is one, wherein at least one and preferably more of the amino acids residues of the parent lipase which occupies a position corresponding to one of the following positions in the *Ps. pseudoalcaligenes* lipase has/have been deleted: G1, L2, F3, G4, S5, T6, G7, I75, S155, T156, S157, L265, T266, S267, L268, F269, E270. The deletion of one or more of the above amino acid residues is believed to result in a variant with an improved wash performance compared to that of the parent enzyme. Preferably, the variant according to this embodiment has been deleted of amino acid residues 1 and 2; of amino acid residues 1-3; of amino acid residues 1-4; of amino acid residues 1-5; or of amino acid residues 1-6.

In another embodiment, the lipase variant of the invention is one which comprises a substitution in an amino acid residue of the parent enzyme, which is located in a position corresponding to one of the following positions in the wild-type *Ps. pseudoalcaligenes* lipase: T6, G7, K12, I16, V18, I27, S39, S42, S46, I49, N56, L60, E64, E67, A73, G79, V84, V93, V110, D118, I126, P128, G132, V136, T156, S157, A161, A164, A175, K179, Y180, I184, A193, K195, S200, V216, S224, D229, P231, H242, K245, D250, D272, T275 and L286.

In a further embodiment the variant of the invention is one, which comprises substitutions in at least the positions corresponding to the following positions in *Ps. pseudoalcaligenes* lipase:

M21+V216+D31+E230;
M21+ S153+S42+P128;
M21+A175+D250;
M21+D229+V216+D31+E230+P128;
M21+S157+E230+V216;
M21+D31+S157+E230+V216;
V216+D31+E230;
S153+S42+P128;
A175+D250;

D229+V216+D31+E230+P128;
S157+E230+V216;
D31+S157+E230+V216;
V216+S224+D229+P231+H242+K245+D250;
L268+D272+T275+L286;
V216+S224+D229+P231+H242+K245+D250+L268+D272+T275+L286;

In another embodiment, the lipase variant is one, wherein an amino acid residue located in a position of the parent lipase, which corresponds to one of the following positions of the *Ps. pseudoalcaligenes* lipase is replaced with an amino acid residue which is hydrophobic and/or positive: I15, M21, I27, L28, D31, W33, Y34, S38, S39, S42, S46, Y48, I49, T50, S53, Q54, L55, L60, L66, E67, V69, I72, A73, S76, G79, V84, V93, Y95, V96, V99, V110, A112, P113, D118, T119, F122, I123, Q125, I126, A131, G132, A134, I135, V136, G138, V140, G142, L146, A161, L162, F176, Y180, Q182, I184, G190, A193, Y194, K195, V196, N197, V199, S200, S208, P209, L210, V216, S217, L219, L220, G222, A223, S225, T227, P231, H242, V259, Q261, T262, T266, S267, L268, V276, Q280, A287 or L289.

Although, in principle any other amino acid residue may be used to replace the above indicated amino acid residue of the parent enzyme it is generally preferred, in relation to the above identified positions, that the substitution to be introduced is a substitution to R, K, W, F, Y, I, L, H, A, V , i.e. a hydrophobic or positive amino acid residue.

When the amino acid residue to be replaced is an E or a D, it is preferably replaced with any of the hydrophobic amino acid residues W, V, F, Y, I, L, A, any of the positively charged amino acid residues R, K or H, or any of the neutral amino acid residues S, T, G or Q.

In a still further embodiment, the lipase variant of the invention is one, wherein one or both of the amino acid residue located in a position of the parent lipase, which corresponds to positions D250 and D272 of the *Ps. pseudoalcaligenes* lipase is replaced with a neutral or positively charged amino acid residue, such as any of the residues disclosed immediately above in connection with replacement of D and E residues.

In a still further embodiment, the lipase variant of the invention is one, wherein an amino acid residue located in a position of the parent lipase, which corresponds to position D25, D31, E51, E63, E67, E70, E71, D118, E166, E171, E191, D229, D250, E258, D272 in the mature, wild-type *Ps. pseudoalcaligenes* lipase has been replaced with a neutral or positively charged amino acid residue as described above.

In a still further embodiment the lipase variant of the invention is one, wherein a negatively charged amino acid residue located in a position of the parent lipase, which corresponds to position D25, D31, D43, E51, E59, E63, E64, E67, E70, E71, D102,

D118, D121, E166, E171, E191, D215, D229, E230, D233, D249, D250, D257, E258 and/or D272 of the mature, wild-type *Ps. pseudoalcaligenes* lipase has been replaced with a neutral or positively charged amino acid residue as described above.

In a still further embodiment the lipase variant of the invention is one, wherein an amino acid residue located at the c-terminal helix of the parent lipase in a position corresponding to position T275, V276, Q279 or L286 of the mature, wild-type *Ps. pseudoalcaligenes* lipase is replaced with a positively charged amino acid residue, particularly R or K.

In a still further embodiment, the lipase variant of the invention is one, wherein an amino acid residue is inserted between the amino acid residues located in a position of the parent lipase, which corresponds to positions 29 and 30; to positions 126 and 127; to positions 188 and 189; to positions193 and 194; and/or to positions 229 and 230 of the wild type *Ps. pseudoalcaligenes* lipase.

In particular, a L may be inserted between the amino acid residues located in a position of the parent lipase, which corresponds to amino acid residues 126 and 127 of the mature *Ps. pseudoalcaligenes* wild-type lipase; an Y between the amino acid residues located in a position of the parent lipase, which corresponds to amino acid residues 188 and 189 of the mature *Ps. pseudoalcaligenes* wild-type lipase; the peptide sequence EVHN between the amino acid residues located in a position of the parent lipase, which corresponds to amino acid residues 193 and194 of the *Ps. pseudoalcaligenes* wild-type lipase; and/or a S between the amino acid residues located in a position of the parent enzyme, which corresponds to amino acid residues 229 and 230 of the *Ps. pseudoalcaligenes* wild-type lipase.

In a preferred embodiment, the lipase variant of the invention comprises at least one, and preferably more of the following substitutions: G1A, G1S, L2W, T6S, G7N, K12Q, I15V, T18V, M21L, M21T, M21V, M21I, L22T, D25N, S26T, I27L, L28G, D31R, D31N, W33F, Y34H, G35T, S38W, S39A, S39N, R41E, S42K, S42R, S46R, S46T, Y48H, I49V, T50A, E51S, S53A, Q54A, L55F, N56D, T57D, L60A, L60Q, E63A, E64K, E64Q, L66A, E67R, E67T, V69I, E70V, E71P, I72W, A73V, S76G, K78G, G79P, V84F, V84I, H88Q, G90S, V93I, V93S, Y95V, V96A, A98S, V99L, V110I, G111N, A112G, P113V, H114N, D118A, D118K, T119V, F122V, I123V, Q125G, I126V, P128E, A131G, G132S, G132I, A134G, I135G, V136A, V136L, A137N, G138A, V140A, G142A, L146V, F149L, S153G, S155N, T156D, T156K, S157T, S157N, S157I, A161S, A161G, L162I, G163N, A164T, E166G, S167T, N169S, N169T, S170T, E171A, A173T, A174S, A175R, F176L, A178S, K179R, Y180F, Y180H, Q182W, I184V, P185N, A188S, G190A, E191K, G192S, A193D, A193T, Y194V, K195R, K195V, V196G, N197H, G198S, V199I, S200R, S205T, T207N, S208A, P209A, L210Y, V216A, V216F, V216P, S217A, L219P, L220F, G222A, A223F, S224T, S225G, T227V, D229G, D229R, D229I, E230F, P231A, P231K,

R238V, S241T, H242R, H242Y, K245M, K245Q, R248D, D250R, D250N, D250S, R252N, L256V, E258A, V259I, Q261H, T262L, L265I, T266R, S267G, L268I, L268P, L268W, D272E, D272S, T275S, V276L, Q280H, L286I, L286N, A287K, and L289V, the respective amino acid residues of the parent enzyme referring to the numbering of the *Ps. pseudoalcaligenes* lipase

In a still further embodiment, the lipase variant of the invention is one, which comprises at least the following substitutions (the numbering referring to the *Ps. pseudoalcaligenes* lipase):

M21L,T,V,I+V216F+D31R,N+E230F;
M21L,T,V,I+ S153G+S42K+P128E;
M21L,T,V,I+A175R+D250R,N;
M21L,T,V,I+D229R,I+V216F+D31R,N+E230F+P128E;
M21L,T,V,I+S157N,I+E230F+V216F;
M21L,T,V,I+D31R,N+S157N,I+E230F+V216F;
V216F+D31R,N+E230F;
S153G+S42K+P128E;
A175R+D250R,N;
D229R,I+V216F+D31R,N+E230F+P128E;
S157N,I+E230F+V216F; or
D31R,N+S157N,I+E230F+V216F.

Furthermore, the variant lipase of the invention may include other modifications of the parent enzyme, in addition to those discussed above. Thus, the lipase variant may be truncated by deleting amino acid residues corresponding to the first 1, 2, 3, 4, 5 or 6 positions at the N-terminal of the *Ps. pseudoalcaligenes* lipase. Also, it may carry a peptide addition at the C-terminal and/or the N-terminal, particularly an extension as disclosed in WO 97/04079, e.g. the extension SPIRR or SPIRPRP at the N-terminal.

The nomenclature used herein for defining mutations is as described in WO 92/05249. For instance, V276L is intended to indicate that the valine located in position 276 of the mature parent lipase is replaced with an L.

As mentioned above the parent lipase to be modified is a lipase belonging to the *Pseudomonas* sp. lipase family defined above. The parent lipase may be a wild-type lipase or a modified lipase carrying one or more amino acid modifications as compared to the wild-type enzyme. In the latter case the mutations disclosed herein is introduced in addition to those of the parent enzyme.

In a preferred embodiment the parent lipase is the *Ps. pseudoalcaligenes* lipase disclosed in EP 334 462 (obtainable from the *Ps. pseudoalcaligenes* strain M1 (CBS 473.85), or WO 95/30744, or a variant of said lipase, or the *Ps. mendocina* SD702 lipase described in WO 95/14783 or a variant of said lipase.

In a further aspect, the invention relates to a DNA construct comprising a DNA sequence encoding a lipase variant of the invention as defined above. The DNA sequence encoding the lipase variant may suitably be prepared by introducing the relevant mutations in a cDNA or genomic DNA sequence encoding the parent lipase. The mutations may be introduced in accordance with well-known techniques such as those disclosed by Sambrook et al. The DNA construct may further comprise control sequences necessary for achieving expression of the modified DNA sequence. The control sequence may be an appropriate promoter sequence, a nucleic acid sequence which is recognized by a host cell for expression of the nucleic acid sequence. The promoter sequence contains transcription and translation control sequences which mediate the expression of the first wash lipolytic enzyme. The promoter may be any nucleic acid sequence which shows transcriptional activity in the host cell of choice and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

The control sequence may also be a suitable transcription terminator sequence, a sequence recognized by a host cell to terminate transcription. The terminator sequence is operably linked to the 3' terminus of the nucleic acid sequence encoding the lipase variant. The terminator sequence may be native to the nucleic acid sequence encoding the lipase variant or may be obtained from foreign sources.

The control sequence may also be a suitable leader sequence, a polyadenylation sequence, a signal peptide encoding sequence, or any other transcriptional or translational regulatory sequence. In addition, the DNA construct may comprise a DNA sequence encoding a factor necessary for producing the lipase variant in active form, a so-called lipase modulator or chaperone, cf. WO 91/00908, WO 93/13200 and EP 331 376.

In a still further aspect the invention relates to an expression vector comprising a DNA construct of the invention as described above. The expression vector may comprise control sequences as described above necessary for the proper expression of the DNA sequence encoding the lipase variant of the invention. The choice of expression vector will depend, e.g., on the host cell intended for use in the production of the lipase. Suitable expression vectors are disclosed, e.g., in WO 91/00908, WO 93/13200, EP 331 376 and WO 95/14783.

In a still further aspect the invention relates to a host cell comprising a DNA construct or an expression vector of the invention. The host cell is preferably a cell of a *Pseudomonas* sp. such as *Ps. pseudoalcaligenes*, or a cell of *E. coli*. In particular, the host cell may be any of the host cells disclosed in WO 91/00908, WO 93/13200, EP 331 376, WO 95/20744 and WO 95/14783.

If no lipase modulator gene is present on the DNA construct or the expression vector of the invention, it is desirable that such gene is present in and capable of being expressed from the host cell of choice so as to enable the production of an active lipase variant from said host cell.

When the host cell is a cell of a *Pseudomonas* sp. it may be desirable that any native lipase gene of such cell is inactivated or deleted. Such inactivation or deletion may be performed in accordance with well-known methods, e.g. as disclosed in WO 95/20744 and WO 9514783. A suitable lipase negative *Pseudomonas* host cell is the *Ps. alcaligenes* PS600 described in WO/9530744 or the lipase negative *Ps. mendocina* strain LD9 described in WO 95/14783.

In a still further aspect the invention relates to a method for producing a lipase variant of the invention comprising (a) cultivating a host cell transformed with a DNA sequence encoding the variant under conditions conducive to expression of the variant; and (b) recovering the variant.

The host cells may be cultivated in a nutrient medium suitable for production of the lipase variant using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentation) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing lipase variant to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art (see, *e.g.*, references for bacteria and yeast; Bennett, J.W. and LaSure, L., editors, *More Gene Manipulations in Fungi*, Academic Press, CA, 1991). Suitable media are available from commercial suppliers or may be prepared according to published compositions (*e.g.*, in catalogues of the American Type Culture Collection). If the lipase variant is secreted into the nutrient medium, the variant can be recovered directly from the medium. If the variant is not secreted, it is recovered from cell lysates.

The resulting lipase variant may be recovered by methods known in the art. For example, the variant may be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation. The recovered variant may then be further purified by a variety of chromatographic procedures, *e.g.*, ion exchange chromatography, gel filtration chromatography, affinity chromatography, or the like.

The lipase variant of the present invention may be purified by a variety of procedures known in the art including, but not limited to, chromatography (*e.g.*, ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (*e.g.*, preparative isoelectric focusing (IEF), differential solubility (*e.g.*,

ammonium sulfate precipitation), or extraction (see, *e.g.*, *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

In accordance with the invention, it is also contemplated to apply, to lipase variant, one or more charged amino acids which permit effective purification of the enzyme. Techniques for doing this is well known by a person skilled in the art of molecular biology.

If no lipase modulator is expressed by the host cell of choice such modulator may be added to the isolated lipase protein or an active lipase may be obtained by use of any other of the methods disclosed in WO 93/13200 or WO 91/00908.

In a still further aspect the invention relates to a method of constructing a variant lipase from a parent lipase belonging to the *Pseudomonas* sp. lipase family, which variant has an improved wash performance as compared to the parent lipase, which method comprises:

subjecting an amino acid subsequence region of the parent lipase which corresponds to one of the following amino acid subsequences 6-14, 15-18, 21-36, 37-39, 46-74, E59-V84, 88-100, 109, 110-136, D118-A145, F149-A173, 154-167, 168-180, A174-S205, 188-200, G206-C239, 222-231, S240-P273, 258-266 or 267-268 of the mature, wild-type *Ps. pseudoalcaligenes* lipase to localized random mutagenesis,

expressing the variety of mutated DNA sequences originating from the parent lipase obtained in step (a) in suitable host cells; and

screening for host cells expressing a mutated lipase which has a decreased dependence on calcium and/or an improved tolerance towards a detergent or a detergent component as compared to the parent lipase.

The localized random mutagenesis performed in step (a) may be essentially as described in WO 95/22615. A preferred method is to use doped or spiked oligonucleotides for the mutagenesis. An example of a doping scheme is given in the Examples hereinafter.

The host cell to be used for expression in step (b) is preferably a cell of *E. coli.*

Preferably, the screening of step (c) is performed for improved tolerance towards an anionic surfactant such as an alkyl sulfate or LAS or a detergent, e.g. the PCS detergent described in the Materials and Methods section herein. The screening is preferably performed by a so-called filter assay, e.g. as described in the Materials and Methods section herein.

It will be understood that once a host cell comprising a mutated DNA sequence encoding a lipase variant with improved wash performance has been identified by the screening step c), the DNA sequence may be isolated from the host cell, and transformed into another host cell to be used for the recombinant production of the

lipase variant. Subsequently, the lipase variant may be prepared and recovered as described hereinbefore.

The lipase variant of the invention may be a hybrid of two lipases belonging to the *Pseudomonas* lipase family having an amino acid sequence with a homology above 40 %. The hybrid lipase will comprise an N-terminal subsequence of one lipase combined with a C-terminal subsequence of the second lipase. Such hybrid lipases may be prepared by methods known in the art, e.g. by module shift using synthetic genes as described in the examples. The hybrid lipases may advantageously include the N-terminal or C-terminal extensions described above.

Examples of suitable lipases for constructing a hybrid are the lipase from *Ps. pseudoalcaligenes* strain M1 described in EP 334,462, the lipase from *Ps. wisconsinensis* described in WO 96/12012, and the lipase SDL-451 derived from *Pseudomonas sp.* SD705 (FERM BP-4772) described in EP 721 981 and WO 96/27002. The amino acid homologies are 44 % between the M1 lipase and the *Ps. wisconsinensis* lipase, 83 % between the M1 lipase and the SDL-451 lipase, and 45 % between the *Ps. wisconsinensis* lipase and the SDL-451 lipase. As demonstrated in the examples, the N-terminal subsequence may terminate at the position corresponding to position S208 of the mature, wild-type *Ps. pseudoalcaligenes* lipase, and the C-terminal subsequence may start at the position corresponding to position P209 of the mature, wild-type *Ps. pseudoalcaligenes* lipase.

In yet another aspect, the invention relates to a method of constructing a variant lipase with improved wash performance from a parent lipase belonging to the *Pseudomonas* lipase family, comprising:

a) selecting a second *Pseudomonas* lipase having an amino acid sequence with a homology above 40 % to that of the parent lipase, preferably above 60 % and more preferably above 80 %,

b) identifying substitutions, insertions and deletions whereby the second sequence differs from the first sequence,

c) designing a variant amino acid sequence by introducing one or more of said substitutions, insertions and deletions into the first amino acid sequence,

d) designing a DNA sequence expressing said variant amino acid sequence,

e) preparing said DNA sequence and transforming a suitable host organism by inserting the DNA sequence,

f) cultivating the transformed host organism to express and secrete a lipase having the variant amino acid sequence and recovering the lipase.

The two amino acid sequences can be aligned by known computer programs like GAP or MegAlign (DNASTAR) to identify sequences with more than 40 % homology. Substitutions, deletions and/or insertions are then identified based on the differences

between the two lipases in the alignment. One or more of the identified substitutions, insertions and deletions can be introduced into the gene encoding the parent *Pseudomonas* lipase by site-directed mutagenesis method or by hybrid gene construction using methods known in the art. The resulting lipase gene may be expressed in *E. coli*, *Pseudomonas*, *Bacillus*, *Aspergillus* or another suitable expression organism and the lipase may be purified and tested for improved wash activity. A number of variants should be tested to find the ones improved in wash tests.

It is an advantage with this method to introduce substitutions, deletions and insertions from a *Pseudomonas* lipase with a known superior wash performance into a homologous *Pseudomonas* lipase with a lower wash performance to improve the wash performance of the latter. An example of a suitable parent lipase is the lipase from *Ps. pseudoalcaligenes* strain M1 described in EP 334,462. An example of a second *Pseudomonas* lipase is the lipase from *Ps. wisconsinensis* described in WO 96/12012. Another example of a second lipase is the lipase SDL-451 derived from *Pseudomonas sp.* SD705 (FERM BP-4772) described in EP 721 981 and WO 96/27002.

The substitutions, deletions and/or insertions to be introduced may advantageously be chosen in the subsequences described above in relation to random mutagenesis. It is preferred to introduce two or more such changes, e.g. three or four changes. Additionally, the variant amino acid sequence may also include the N-terminal or C-terminal extensions described above, the sequence may further be subjected to random mutagenesis as described above, and the sequence may additinoally include one or more of the substitutions, deletions or insertions described earlier in this specification.

In a final aspect the invention relates to a detergent composition comprising a lipase variant of the invention.

DETERGENT DISCLOSURE AND EXAMPLES

Surfactant system

The detergent compositions according to the present invention comprise a surfactant system, wherein the surfactant can be selected from nonionic and/or anionic and/or cationic and/or ampholytic and/or zwitterionic and/or semi-polar surfactants.

The surfactant is typically present at a level from 0.1% to 60% by weight.

The surfactant is preferably formulated to be compatible with enzyme components present in the composition. In liquid or gel compositions the surfactant is most preferably formulated in such a way that it promotes, or at least does not degrade, the stability of any enzyme in these compositions.

Preferred systems to be used according to the present invention comprise as a surfactant one or more of the nonionic and/or anionic surfactants described herein.

Polyethylene, polypropylene, and polybutylene oxide condensates of alkyl phenols are suitable for use as the nonionic surfactant of the surfactant systems of the present invention, with the polyethylene oxide condensates being preferred. These compounds include the condensation products of alkyl phenols having an alkyl group containing from about 6 to about 14 carbon atoms, preferably from about 8 to about 14 carbon atoms, in either a straight chain or branched-chain configuration with the alkylene oxide. In a preferred embodiment, the ethylene oxide is present in an amount equal to from about 2 to about 25 moles, more preferably from about 3 to about 15 moles, of ethylene oxide per mole of alkyl phenol. Commercially available nonionic surfactants of this type include Igepal™ CO-630, marketed by the GAF Corporation; and Triton™ X-45, X-114, X-100 and X-102, all marketed by the Rohm & Haas Company. These surfactants are commonly referred to as alkylphenol alkoxylates (e.g., alkyl phenol ethoxylates).

The condensation products of primary and secondary aliphatic alcohols with about 1 to about 25 moles of ethylene oxide are suitable for use as the nonionic surfactant of the nonionic surfactant systems of the present invention. The alkyl chain of the aliphatic alcohol can either be straight or branched, primary or secondary, and generally contains from about 8 to about 22 carbon atoms. Preferred are the condensation products of alcohols having an alkyl group containing from about 8 to about 20 carbon atoms, more preferably from about 10 to about 18 carbon atoms, with from about 2 to about 10 moles of ethylene oxide per mole of alcohol. About 2 to about 7 moles of ethylene oxide and most preferably from 2 to 5 moles of ethylene oxide per mole of alcohol are present in said condensation products. Examples of commercially available nonionic surfactants of this type include Tergitol™ 15-S-9 (The condensation product of C_{11}-C_{15} linear alcohol with 9 moles ethylene oxide), Tergitol™ 24-L-6 NMW (the condensation product of C_{12}-C_{14} primary alcohol with 6 moles ethylene oxide with a narrow molecular weight distribution), both marketed by Union Carbide Corporation; Neodol™ 45-9 (the condensation product of C_{14}-C_{15} linear alcohol with 9 moles of ethylene oxide), Neodol™ 23-3 (the condensation product of C_{12}-C_{13} linear alcohol with 3.0 moles of ethylene oxide), Neodol™ 45-7 (the condensation product of C_{14}-C_{15} linear alcohol with 7 moles of ethylene oxide), Neodol™ 45-5 (the condensation product of C_{14}-C_{15} linear alcohol with 5 moles of ethylene oxide) marketed by Shell Chemical Company, Kyro™ EOB (the condensation product of C_{13}-C_{15} alcohol with 9 moles ethylene oxide), marketed by The Procter & Gamble Company, and Genapol LA 050 (the condensation product of C_{12}-C_{14} alcohol with 5 moles of ethylene oxide) marketed by Hoechst. Preferred range of HLB in these products is from 8-11 and most preferred from 8-10.

Also useful as the nonionic surfactant of the surfactant systems of the present invention are alkylpolysaccharides disclosed in US 4,565,647, having a hydrophobic group containing from about 6 to about 30 carbon atoms, preferably from about 10 to about 16 carbon atoms and a polysaccharide, e.g. a polyglycoside, hydrophilic group containing from about 1.3 to about 10, preferably from about 1.3 to about 3, most preferably from about 1.3 to about 2.7 saccharide units. Any reducing saccharide containing 5 or 6 carbon atoms can be used, e.g., glucose, galactose and galactosyl moieties can be substituted for the glucosyl moieties (optionally the hydrophobic group is attached at the 2-, 3-, 4-, etc. positions thus giving a glucose or galactose as opposed to a glucoside or galactoside). The intersaccharide bonds can be, e.g., between the one position of the additional saccharide units and the 2-, 3-, 4-, and/or 6- positions on the preceding saccharide units.

The preferred alkylpolyglycosides have the formula

$$R^2O(C_nH_{2n}O)_t(\text{glycosyl})_x$$

wherein R^2 is selected from the group consisting of alkyl, alkylphenyl, hydroxyalkyl, hydroxyalkylphenyl, and mixtures thereof in which the alkyl groups contain from about 10 to about 18, preferably from about 12 to about 14, carbon atoms; n is 2 or 3, preferably 2; t is from 0 to about 10, preferably 0; and x is from about 1.3 to about 10, preferably from about 1.3 to about 3, most preferably from about 1.3 to about 2.7. The glycosyl is preferably derived from glucose. To prepare these compounds, the alcohol or alkylpolyethoxy alcohol is formed first and then reacted with glucose, or a source of glucose, to form the glucoside (attachment at the 1-position). The additional glycosyl units can then be attached between their 1-position and the preceding glycosyl units 2-, 3-, 4-, and/or 6-position, preferably predominantly the 2-position.

The condensation products of ethylene oxide with a hydrophobic base formed by the condensation of propylene oxide with propylene glycol are also suitable for use as the additional nonionic surfactant systems of the present invention. The hydrophobic portion of these compounds will preferably have a molecular weight from about 1500 to about 1800 and will exhibit water insolubility. The addition of polyoxyethylene moieties to this hydrophobic portion tends to increase the water solubility of the molecule as a whole, and the liquid character of the product is retained up to the point where the polyoxyethylene content is about 50% of the total weight of the condensation product, which corresponds to condensation with up to about 40 moles of ethylene oxide. Examples of compounds of this type include certain of the commercially available Pluronic™ surfactants, marketed by BASF.

Also suitable for use as the nonionic surfactant of the nonionic surfactant system of the present invention, are the condensation products of ethylene oxide with the product resulting from the reaction of propylene oxide and ethylenediamine. The hydrophobic moiety of these products consists of the reaction product of ethylenediamine and excess propylene oxide, and generally has a molecular weight of from about 2500 to about 3000. This hydrophobic moiety is condensed with ethylene oxide to the extent that the condensation product contains from about 40% to about 80% by weight of polyoxyethylene and has a molecular weight of from about 5,000 to about 11,000. Examples of this type of nonionic surfactant include certain of the commercially available Tetronic™ compounds, marketed by BASF.

Preferred for use as the nonionic surfactant of the surfactant systems of the present invention are polyethylene oxide condensates of alkyl phenols, condensation products of primary and secondary aliphatic alcohols with from about 1 to about 25 moles of ethylene oxide, alkylpolysaccharides, and mixtures hereof. Most preferred are C_8-C_{14} alkyl phenol ethoxylates having from 3 to 15 ethoxy groups and C_8-C_{18} alcohol ethoxylates (preferably C_{10} avg.) having from 2 to 10 ethoxy groups, and mixtures thereof.

Highly preferred nonionic surfactants are polyhydroxy fatty acid amide surfactants of the formula

$$R^2-\underset{\underset{O}{\|}}{C}-\underset{\underset{R^1}{|}}{N}-Z,$$

wherein R^1 is H, or R^1 is C_{1-4} hydrocarbyl, 2-hydroxyethyl, 2-hydroxypropyl or a mixture thereof, R^2 is C_{5-31} hydrocarbyl, and Z is a polyhydroxyhydrocarbyl having a linear hydrocarbyl chain with at least 3 hydroxyls directly connected to the chain, or an alkoxylated derivative thereof. Preferably, R^1 is methyl, R^2 is straight C_{11-15} alkyl or C_{16-18} alkyl or alkenyl chain such as coconut alkyl or mixtures thereof, and Z is derived from a reducing sugar such as glucose, fructose, maltose or lactose, in a reductive amination reaction.

Highly preferred anionic surfactants include alkyl alkoxylated sulfate surfactants. Examples hereof are water soluble salts or acids of the formula $RO(A)_mSO3M$ wherein R is an unsubstituted C_{10}-C-$_{24}$ alkyl or hydroxyalkyl group having a C_{10}-C_{24} alkyl component, preferably a C_{12}-C_{20} alkyl or hydroxyalkyl, more preferably C_{12}-C_{18} alkyl or hydroxyalkyl, A is an ethoxy or propoxy unit, m is greater than zero, typically between about 0.5 and about 6, more preferably between about 0.5 and about 3, and M is H or a cation which can be, for example, a metal cation (e.g., sodium, potassium, lithium, calcium, magnesium, etc.), ammonium or substituted-ammonium cation. Alkyl ethoxylated sulfates as well as alkyl propoxylated sulfates are contemplated herein. Specific

examples of substituted ammonium cations include methyl-, dimethyl, trimethyl-ammonium cations and quaternary ammonium cations such as tetramethyl-ammonium and dimethyl piperdinium cations and those derived from alkylamines such as ethylamine, diethylamine, triethylamine, mixtures thereof, and the like. Exemplary surfactants are C_{12}-C_{18} alkyl polyethoxylate (1.0) sulfate (C_{12}-C_{18}E(1.0)M), C_{12}-C_{18} alkyl polyethoxylate (2.25) sulfate (C_{12}-C_{18}(2.25)M, and C_{12}-C_{18} alkyl polyethoxylate (3.0) sulfate (C_{12}-C_{18}E(3.0)M), and C_{12}-C_{18} alkyl polyethoxylate (4.0) sulfate (C_{12}-C_{18}E(4.0)M), wherein M is conveniently selected from sodium and potassium.

Suitable anionic surfactants to be used are alkyl ester sulfonate surfactants including linear esters of C_8-C_{20} carboxylic acids (i.e., fatty acids) which are sulfonated with gaseous SO_3 according to "The Journal of the American Oil Chemists Society", 52 (1975), pp. 323-329. Suitable starting materials would include natural fatty substances as derived from tallow, palm oil, etc.

The preferred alkyl ester sulfonate surfactant, especially for laundry applications, comprise alkyl ester sulfonate surfactants of the structural formula:

$$R^3-\underset{\displaystyle SO_3M}{\underset{|}{CH}}-\overset{\displaystyle O}{\overset{\|}{C}}-OR^4$$

wherein R^3 is a C_8-C_{20} hydrocarbyl, preferably an alkyl, or combination thereof, R^4 is a C_1-C_6 hydrocarbyl, preferably an alkyl, or combination thereof, and M is a cation which forms a water soluble salt with the alkyl ester sulfonate. Suitable salt-forming cations include metals such as sodium, potassium, and lithium, and substituted or unsubstituted ammonium cations, such as monoethanolamine, diethonolamine, and triethanolamine. Preferably, R^3 is C_{10}-C_{16} alkyl, and R^4 is methyl, ethyl or isopropyl. Especially preferred are the methyl ester sulfonates wherein R^3 is C_{10}-C_{16} alkyl.

Other suitable anionic surfactants include the alkyl sulfate surfactants which are water soluble salts or acids of the formula $ROSO_3M$ wherein R preferably is a C_{10}-C_{24} hydrocarbyl, preferably an alkyl or hydroxyalkyl having a C_{10}-C_{20} alkyl component, more preferably a C_{12}-C_{18} alkyl or hydroxyalkyl, and M is H or a cation, e.g., an alkali metal cation (e.g. sodium, potassium, lithium), or ammonium or substituted ammonium (e.g. methyl-, dimethyl-, and trimethyl ammonium cations and quaternary ammonium cations such as tetramethyl-ammonium and dimethyl piperdinium cations and quaternary ammonium cations derived from alkylamines such as ethylamine, diethylamine, triethylamine, and mixtures thereof, and the like). Typically, alkyl chains of C_{12}-C_{16} are

preferred for lower wash temperatures (e.g. below about 50°C) and C_{16}-C_{18} alkyl chains are preferred for higher wash temperatures (e.g. above about 50°C).

Other anionic surfactants useful for detersive purposes can also be included in the laundry detergent compositions of the present invention. Theses can include salts (including, for example, sodium, potassium, ammonium, and substituted ammonium salts such as mono- di- and triethanolamine salts) of soap, C_8-C_{22} primary or secondary alkanesulfonates, C_8-C_{24} olefinsulfonates, sulfonated polycarboxylic acids prepared by sulfonation of the pyrolyzed product of alkaline earth metal citrates, e.g., as described in British patent specification No. 1,082,179, C_8-C_{24} alkylpolyglycolethersulfates (containing up to 10 moles of ethylene oxide); alkyl glycerol sulfonates, fatty acyl glycerol sulfonates, fatty oleyl glycerol sulfates, alkyl phenol ethylene oxide ether sulfates, paraffin sulfonates, alkyl phosphates, isethionates such as the acyl isethionates, N-acyl taurates, alkyl succinamates and sulfosuccinates, monoesters of sulfosuccinates (especially saturated and unsaturated C_{12}-C_{18} monoesters) and diesters of sulfosuccinates (especially saturated and unsaturated C_6-C_{12} diesters), acyl sarcosinates, sulfates of alkylpolysaccharides such as the sulfates of alkylpolyglucoside (the nonionic nonsulfated compounds being described below), branched primary alkyl sulfates, and alkyl polyethoxy carboxylates such as those of the formula $RO(CH_2CH_2O)_k$-CH_2C00-M+ wherein R is a C_8-C_{22} alkyl, k is an integer from 1 to 10, and M is a soluble salt forming cation. Resin acids and hydrogenated resin acids are also suitable, such as rosin, hydrogenated rosin, and resin acids and hydrogenated resin acids present in or derived from tall oil.

Alkylbenzene sulfonates are highly preferred. Especially preferred are linear (straight-chain) alkyl benzene sulfonates (LAS) wherein the alkyl group preferably contains from 10 to 18 carbon atoms.

Further examples are described in "Surface Active Agents and Detergents" (Vol. I and II by Schwartz, Perry and Berch). A variety of such surfactants are also generally disclosed in US 3,929,678, (Column 23, line 58 through Column 29, line 23, herein incorporated by reference).

When included therein, the laundry detergent compositions of the present invention typically comprise from about 1% to about 40%, preferably from about 3% to about 20% by weight of such anionic surfactants.

The laundry detergent compositions of the present invention may also contain cationic, ampholytic, zwitterionic, and semi-polar surfactants, as well as the nonionic and/or anionic surfactants other than those already described herein.

Cationic detersive surfactants suitable for use in the laundry detergent compositions of the present invention are those having one long-chain hydrocarbyl group.

Examples of such cationic surfactants include the ammonium surfactants such as alkyltrimethylammonium halogenides, and those surfactants having the formula:

$[R^2(OR^3)_y][R^4(OR^3)_y]_2R^5N+X-$

wherein R^2 is an alkyl or alkyl benzyl group having from about 8 to about 18 carbon atoms in the alkyl chain, each R^3 is selected form the group consisting of -CH_2CH_2-, -$CH_2CH(CH_3)$-, -$CH_2CH(CH_2OH)$-, -$CH_2CH_2CH_2$-, and mixtures thereof; each R^4 is selected from the group consisting of C_1-C_4 alkyl, C_1-C_4 hydroxyalkyl, benzyl ring structures formed by joining the two R^4 groups, -$CH_2CHOHCHOHCOR^6CHOHCH_2OH$, wherein R^6 is any hexose or hexose polymer having a molecular weight less than about 1000, and hydrogen when y is not 0; R^5 is the same as R^4 or is an alkyl chain, wherein the total number of carbon atoms or R^2 plus R^5 is not more than about 18; each y is from 0 to about 10,and the sum of the y values is from 0 to about 15; and X is any compatible anion.

Highly preferred cationic surfactants are the water soluble quaternary ammonium compounds useful in the present composition having the formula:

$R_1R_2R_3R_4N^+X^-$ (i)

wherein R_1 is C_8-C_{16} alkyl, each of R_2, R_3 and R_4 is independently C_1-C_4 alkyl, C_1-C_4 hydroxy alkyl, benzyl, and -$(C_2H_{40})_xH$ where x has a value from 2 to 5, and X is an anion. Not more than one of R_2, R_3 or R_4 should be benzyl.

The preferred alkyl chain length for R_1 is C_{12}-C_{15}, particularly where the alkyl group is a mixture of chain lengths derived from coconut or palm kernel fat or is derived synthetically by olefin build up or OXO alcohols synthesis.

Preferred groups for R_2R_3 and R_4 are methyl and hydroxyethyl groups and the anion X may be selected from halide, methosulphate, acetate and phosphate ions.

Examples of suitable quaternary ammonium compounds of formulae (i) for use herein are:

coconut trimethyl ammonium chloride or bromide;
coconut methyl dihydroxyethyl ammonium chloride or bromide;
decyl triethyl ammonium chloride;
decyl dimethyl hydroxyethyl ammonium chloride or bromide;
C_{12-15} dimethyl hydroxyethyl ammonium chloride or bromide;
coconut dimethyl hydroxyethyl ammonium chloride or bromide;
myristyl trimethyl ammonium methyl sulphate;
lauryl dimethyl benzyl ammonium chloride or bromide;

lauryl dimethyl (ethenoxy)$_4$ ammonium chloride or bromide;

choline esters (compounds of formula (i) wherein R_1 is

$$CH_2\text{-}CH_2\text{-}O\text{-}\underset{\underset{O}{\|}}{C}\text{-}C_{12\text{-}14}\text{ alkyl and } R_2R_3R_4 \text{ are methyl).}$$

di-alkyl imidazolines [compounds of formula (i)].

Other cationic surfactants useful herein are also described in US 4,228,044 and in EP 000 224.

When included therein, the laundry detergent compositions of the present invention typically comprise from 0.2% to about 25%, preferably from about 1% to about 8% by weight of such cationic surfactants.

Ampholytic surfactants are also suitable for use in the laundry detergent compositions of the present invention. These surfactants can be broadly described as aliphatic derivatives of secondary or tertiary amines, or aliphatic derivatives of heterocyclic secondary and tertiary amines in which the aliphatic radical can be straight- or branched-chain. One of the aliphatic substituents contains at least about 8 carbon atoms, typically from about 8 to about 18 carbon atoms, and at least one contains an anionic water-solubilizing group, e.g. carboxy, sulfonate, sulfate. See US 3,929,678 (column 19, lines 18-35) for examples of ampholytic surfactants.

When included therein, the laundry detergent compositions of the present invention typically comprise from 0.2% to about 15%, preferably from about 1% to about 10% by weight of such ampholytic surfactants.

Zwitterionic surfactants are also suitable for use in laundry detergent compositions. These surfactants can be broadly described as derivatives of secondary and tertiary amines, derivatives of heterocyclic secondary and tertiary amines, or derivatives of quaternary ammonium, quaternary phosphonium or tertiary sulfonium compounds. See US 3,929,678 (column 19, line 38 through column 22, line 48) for examples of zwitterionic surfactants.

When included therein, the laundry detergent compositions of the present invention typically comprise from 0.2% to about 15%, preferably from about 1% to about 10% by weight of such zwitterionic surfactants.

Semi-polar nonionic surfactants are a special category of nonionic surfactants which include water-soluble amine oxides containing one alkyl moiety of from about 10 to about 18 carbon atoms and 2 moieties selected from the group consisting of alkyl groups and hydroxyalkyl groups containing from about 1 to about 3 carbon atoms; water-soluble phosphine oxides containing one alkyl moiety of from about 10 to about 18

carbon atoms and 2 moieties selected from the group consisting of alkyl groups and hydroxyalkyl groups containing from about 1 to about 3 carbon atoms; and water-soluble sulfoxides containing one alkyl moiety from about 10 to about 18 carbon atoms and a moiety selected from the group consisting of alkyl and hydroxyalkyl moieties of from about 1 to about 3 carbon atoms.

Semi-polar nonionic detergent surfactants include the amine oxide surfactants having the formula:

$$R^3(OR^4)_xN(R^5)_2 \text{ with } \overset{O}{\uparrow} \text{ on N}$$

wherein R^3 is an alkyl, hydroxyalkyl, or alkyl phenyl group or mixtures thereof containing from about 8 to about 22 carbon atoms; R^4 is an alkylene or hydroxyalkylene group containing from about 2 to about 3 carbon atoms or mixtures thereof; x is from 0 to about 3: and each R^5 is an alkyl or hydroxyalkyl group containing from about 1 to about 3 carbon atoms or a polyethylene oxide group containing from about 1 to about 3 ethylene oxide groups. The R^5 groups can be attached to each other, e.g., through an oxygen or nitrogen atom, to form a ring structure.

These amine oxide surfactants in particular include C_{10}-C_{18} alkyl dimethyl amine oxides and C_8-C_{12} alkoxy ethyl dihydroxy ethyl amine oxides.

When included therein, the laundry detergent compositions of the present invention typically comprise from 0.2% to about 15%, preferably from about 1% to about 10% by weight of such semi-polar nonionic surfactants.

Builder system

The compositions according to the present invention may further comprise a builder system. Any conventional builder system is suitable for use herein including aluminosilicate materials, silicates, polycarboxylates and fatty acids, materials such as ethylenediamine tetraacetate, metal ion sequestrants such as aminopolyphos-phonates, particularly ethylenediamine tetramethylene phosphonic acid and diethylene triamine pentamethylenephosphonic acid. Though less preferred for obvious environmental reasons, phosphate builders can also be used herein.

Suitable builders can be an inorganic ion exchange material, commonly an inorganic hydrated aluminosilicate material, more particularly a hydrated synthetic zeolite such as hydrated zeolite A, X, B, HS or MAP.

Another suitable inorganic builder material is layered silicate, e.g. SKS-6 (Hoechst). SKS-6 is a crystalline layered silicate consisting of sodium silicate ($Na_2Si_2O_5$).

Suitable polycarboxylates containing one carboxy group include lactic acid, glycolic acid and ether derivatives thereof as disclosed in Belgian Patent Nos. 831,368, 821,369 and 821,370. Polycarboxylates containing two carboxy groups include the water-soluble salts of succinic acid, malonic acid, (ethylenedioxy) diacetic acid, maleic acid, diglycollic acid, tartaric acid, tartronic acid and fumaric acid, as well as the ether carboxylates described in German Offenlegenschrift 2,446,686, and 2,446,487, US 3,935,257 and the sulfinyl carboxylates described in Belgian Patent No. 840,623. Polycarboxylates containing three carboxy groups include, in particular, water-soluble citrates, aconitrates and citraconates as well as succinate derivatives such as the carboxymethyloxysuccinates described in British Patent No. 1,379,241, lactoxysuccinates described in Netherlands Application 7205873, and the oxypolycarboxylate materials such as 2-oxa-1,1,3-propane tricarboxylates described in British Patent No. 1,387,447.

Polycarboxylates containing four carboxy groups include oxydisuccinates disclosed in British Patent No. 1,261,829, 1,1,2,2,-ethane tetracarboxylates, 1,1,3,3-propane tetracarboxylates containing sulfo substituents include the sulfosuccinate derivatives disclosed in British Patent Nos. 1,398,421 and 1,398,422 and in US 3,936,448, and the sulfonated pyrolysed citrates described in British Patent No. 1,082,179, while polycarboxylates containing phosphone substituents are disclosed in British Patent No. 1,439,000.

Alicyclic and heterocyclic polycarboxylates include cyclopentane-cis,cis-cis-tetracarboxylates, cyclopentadienide pentacarboxylates, 2,3,4,5-tetrahydro-furan - cis, cis, cis-tetracarboxylates, 2,5-tetrahydro-furan-cis, discarboxylates, 2,2,5,5,-tetrahydrofuran - tetracarboxylates, 1,2,3,4,5,6-hexane - hexacarboxylates and carboxymethyl derivatives of polyhydric alcohols such as sorbitol, mannitol and xylitol. Aromatic polycarboxylates include mellitic acid, pyromellitic acid and the phthalic acid derivatives disclosed in British Patent No. 1,425,343.

Of the above, the preferred polycarboxylates are hydroxy-carboxylates containing up to three carboxy groups per molecule, more particularly citrates.

Preferred builder systems for use in the present compositions include a mixture of a water-insoluble aluminosilicate builder such as zeolite A or of a layered silicate (SKS-6), and a water-soluble carboxylate chelating agent such as citric acid.

A suitable chelant for inclusion in the detergent compositions in accordance with the invention is ethylenediamine-N,N'-disuccinic acid (EDDS) or the alkali metal, alkaline earth metal, ammonium, or substituted ammonium salts thereof, or mixtures thereof. Preferred EDDS compounds are the free acid form and the sodium or magnesium salt thereof. Examples of such preferred sodium salts of EDDS include Na_2EDDS and Na_4EDDS. Examples of such preferred magnesium salts of EDDS include MgEDDS and

Mg_2EDDS. The magnesium salts are the most preferred for inclusion in compositions in accordance with the invention.

Preferred builder systems include a mixture of a water-insoluble aluminosilicate builder such as zeolite A, and a water soluble carboxylate chelating agent such as citric acid.

Other builder materials that can form part of the builder system for use in granular compositions include inorganic materials such as alkali metal carbonates, bicarbonates, silicates, and organic materials such as the organic phosphonates, amino polyalkylene phosphonates and amino polycarboxylates.

Other suitable water-soluble organic salts are the homo- or co-polymeric acids or their salts, in which the polycarboxylic acid comprises at least two carboxyl radicals separated form each other by not more than two carbon atoms.

Polymers of this type are disclosed in GB-A-1,596,756. Examples of such salts are polyacrylates of MW 2000-5000 and their copolymers with maleic anhydride, such copolymers having a molecular weight of from 20,000 to 70,000, especially about 40,000.

Detergency builder salts are normally included in amounts of from 5% to 80% by weight of the composition. Preferred levels of builder for liquid detergents are from 5% to 30%.

Enzymes

Preferred detergent compositions, in addition to the enzyme preparation of the invention, comprise other enzyme(s) which provides cleaning performance and/or fabric care benefits.

Such enzymes include proteases, lipases, cutinases, amylases, cellulases, peroxidases, oxidases (e.g. laccases).

Proteases:

Any protease suitable for use in alkaline solutions can be used. Suitable proteases include those of animal, vegetable or microbial origin. Microbial origin is preferred. Chemically or genetically modified mutants are included. The protease may be a serine protease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from Bacillus, e.g., subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (described in WO 89/06279). Examples of trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the Fusarium protease described in WO 89/06270.

Preferred commercially available protease enzymes include those sold under the trade names Alcalase, Savinase, Primase, Durazym, and Esperase by Novo Nordisk

A/S (Denmark), those sold under the trade name Maxatase, Maxacal, Maxapem, Properase, Purafect and Purafect OXP by Genencor International, and those sold under the trade name Opticlean and Optimase by Solvay Enzymes. Protease enzymes may be incorporated into the compositions in accordance with the invention at a level of from 0.00001% to 2% of enzyme protein by weight of the composition, preferably at a level of from 0.0001% to 1% of enzyme protein by weight of the composition, more preferably at a level of from 0.001% to 0.5% of enzyme protein by weight of the composition, even more preferably at a level of from 0.01% to 0.2% of enzyme protein by weight of the composition.

Lipases:

Any lipase suitable for use in alkaline solutions can be used. Suitable lipases include those of bacterial or fungal origin. Chemically or genetically modified mutants are included.

Examples of useful lipases include a Humicola lanuginosa lipase, e.g., as described in EP 258 068 and EP 305 216, a Rhizomucor miehei lipase, e.g., as described in EP 238 023, a Candida lipase, such as a C. antarctica lipase, e.g., the C. antarctica lipase A or B described in EP 214 761, a Pseudomonas lipase such as a P. alcaligenes and P. pseudoalcaligenes lipase, e.g., as described in EP 218 272, a P. cepacia lipase, e.g., as described in EP 331 376, a P. stutzeri lipase, e.g., as disclosed in GB 1,372,034, a P. fluorescens lipase, a Bacillus lipase, e.g., a B. subtilis lipase (Dartois et al., (1993), Biochemica et Biophysica acta 1131, 253-260), a B. stearothermophilus lipase (JP 64/744992) and a B. pumilus lipase (WO 91/16422).

Furthermore, a number of cloned lipases may be useful, including the Penicillium camembertii lipase described by Yamaguchi et al., (1991), Gene 103, 61-67), the Geotrichum candidum lipase (Schimada, Y. et al., (1989), J. Biochem., 106, 383-388), and various Rhizopus lipases such as a R. delemar lipase (Hass, M.J et al., (1991), Gene 109, 117-113), a R. niveus lipase (Kugimiya et al., (1992), Biosci. Biotech. Biochem. 56, 716-719) and a R. oryzae lipase.

Other types of lipolytic enzymes such as cutinases may also be useful, e.g., a cutinase derived from Pseudomonas mendocina as described in WO 88/09367, or a cutinase derived from Fusarium solani pisi (e.g. described in WO 90/09446).

Especially suitable lipases are lipases such as M1 Lipase™, Luma fast™ and Lipomax™ (Genencor), Lipolase™ and Lipolase Ultra™ (Novo Nordisk A/S), and Lipase P "Amano" (Amano Pharmaceutical Co. Ltd.).

The lipases are normally incorporated in the detergent composition at a level of from 0.00001% to 2% of enzyme protein by weight of the composition, preferably at a level of from 0.0001% to 1% of enzyme protein by weight of the composition, more

preferably at a level of from 0.001% to 0.5% of enzyme protein by weight of the composition, even more preferably at a level of from 0.01% to 0.2% of enzyme protein by weight of the composition.

Amylases:

Any amylase (α and/or β) suitable for use in alkaline solutions can be used. Suitable amylases include those of bacterial or fungal origin. Chemically or genetically modified mutants are included. Amylases include, for example, α-amylases obtained from a special strain of B. licheniformis, described in more detail in GB 1,296,839. Commercially available amylases are Duramyl™, Termamyl™, Fungamyl™ and BAN™ (available from Novo Nordisk A/S) and Rapidase™ and Maxamyl P™ (available from Genencor).

The amylases are normally incorporated in the detergent composition at a level of from 0.00001% to 2% of enzyme protein by weight of the composition, preferably at a level of from 0.0001% to 1% of enzyme protein by weight of the composition, more preferably at a level of from 0.001% to 0.5% of enzyme protein by weight of the composition, even more preferably at a level of from 0.01% to 0.2% of enzyme protein by weight of the composition.

Cellulases:

Any cellulase suitable for use in alkaline solutions can be used. Suitable cellulases include those of bacterial or fungal origin. Chemically or genetically modified mutants are included. Suitable cellulases are disclosed in US 4,435,307, which discloses fungal cellulases produced from Humicola insolens. Especially suitable cellulases are the cellulases having color care benefits. Examples of such cellulases are cellulases described in European patent application No. 0 495 257.

Commercially available cellulases include Celluzyme™ produced by a strain of Humicola insolens, (Novo Nordisk A/S), and KAC-500(B)™ (Kao Corporation).

Cellulases are normally incorporated in the detergent composition at a level of from 0.00001% to 2% of enzyme protein by weight of the composition, preferably at a level of from 0.0001% to 1% of enzyme protein by weight of the composition, more preferably at a level of from 0.001% to 0.5% of enzyme protein by weight of the composition, even more preferably at a level of from 0.01% to 0.2% of enzyme protein by weight of the composition.

Peroxidases/Oxidases:

Peroxidase enzymes are used in combination with hydrogen peroxide or a source thereof (e.g. a percarbonate, perborate or persulfate). Oxidase enzymes are

used in combination with oxygen. Both types of enzymes are used for "solution bleaching", i.e. to prevent transfer of a textile dye from a dyed fabric to another fabric when said fabrics are washed together in a wash liquor, preferably together with an enhancing agent as described in e.g. WO 94/12621 and WO 95/01426. Suitable peroxidases/oxidases include those of plant, bacterial or fungal origin. Chemically or genetically modified mutants are included.

Peroxidase and/or oxidase enzymes are normally incorporated in the detergent composition at a level of from 0.00001% to 2% of enzyme protein by weight of the composition, preferably at a level of from 0.0001% to 1% of enzyme protein by weight of the composition, more preferably at a level of from 0.001% to 0.5% of enzyme protein by weight of the composition, even more preferably at a level of from 0.01% to 0.2% of enzyme protein by weight of the composition.

Mixtures of the above mentioned enzymes are encompassed herein, in particular a mixture of a protease, an amylase, a lipase and/or a cellulase.

The enzyme of the invention, or any other enzyme incorporated in the detergent composition, is normally incorporated in the detergent composition at a level from 0.00001% to 2% of enzyme protein by weight of the composition, preferably at a level from 0.0001% to 1% of enzyme protein by weight of the composition, more preferably at a level from 0.001% to 0.5% of enzyme protein by weight of the composition, even more preferably at a level from 0.01% to 0.2% of enzyme protein by weight of the composition.

Bleaching agents:

Additional optional detergent ingredients that can be included in the detergent compositions of the present invention include bleaching agents such as PB1, PB4 and percarbonate with a particle size of 400-800 microns. These bleaching agent components can include one or more oxygen bleaching agents and, depending upon the bleaching agent chosen, one or more bleach activators. When present oxygen bleaching compounds will typically be present at levels of from about 1% to about 25%. In general, bleaching compounds are optional added components in non-liquid formulations, e.g. granular detergents.

The bleaching agent component for use herein can be any of the bleaching agents useful for detergent compositions including oxygen bleaches as well as others known in the art.

The bleaching agent suitable for the present invention can be an activated or non-activated bleaching agent.

One category of oxygen bleaching agent that can be used encompasses percarboxylic acid bleaching agents and salts thereof. Suitable examples of this class of agents include magnesium monoperoxyphthalate hexahydrate, the magnesium salt of

meta-chloro perbenzoic acid, 4-nonylamino-4-oxoperoxybutyric acid and diperoxy-dodecanedioic acid. Such bleaching agents are disclosed in US 4,483,781, US 740,446, EP 0 133 354 and US 4,412,934. Highly preferred bleaching agents also include 6-nonylamino-6-oxoperoxycaproic acid as described in US 4,634,551.

Another category of bleaching agents that can be used encompasses the halogen bleaching agents. Examples of hypohalite bleaching agents, for example, include trichloro isocyanuric acid and the sodium and potassium dichloroisocyanurates and N-chloro and N-bromo alkane sulphonamides. Such materials are normally added at 0.5-10% by weight of the finished product, preferably 1-5% by weight.

The hydrogen peroxide releasing agents can be used in combination with bleach activators such as tetra-acetylethylenediamine (TAED), nonanoyloxybenzene-sulfonate (NOBS, described in US 4,412,934), 3,5-trimethyl-hexsanoloxybenzene-sulfonate (ISONOBS, described in EP 120 591) or pentaacetylglucose (PAG), which are perhydrolyzed to form a peracid as the active bleaching species, leading to improved bleaching effect. In addition, very suitable are the bleach activators C8(6-octanamido-caproyl) oxybenzene-sulfonate, C9(6-nonanamido caproyl) oxybenzene-sulfonate and C10 (6-decanamido caproyl) oxybenzenesulfonate or mixtures thereof. Also suitable activators are acylated citrate esters such as disclosed in European Patent Application No. 91870207.7.

Useful bleaching agents, including peroxyacids and bleaching systems comprising bleach activators and peroxygen bleaching compounds for use in cleaning compositions according to the invention are described in application USSN 08/136,626.

The hydrogen peroxide may also be present by adding an enzymatic system (i.e. an enzyme and a substrate therefore) which is capable of generation of hydrogen peroxide at the beginning or during the washing and/or rinsing process. Such enzymatic systems are disclosed in European Patent Application EP 0 537 381.

Bleaching agents other than oxygen bleaching agents are also known in the art and can be utilized herein. One type of non-oxygen bleaching agent of particular interest includes photoactivated bleaching agents such as the sulfonated zinc and/or aluminum phthalocyanines. These materials can be deposited upon the substrate during the washing process. Upon irradiation with light, in the presence of oxygen, such as by hanging clothes out to dry in the daylight, the sulfonated zinc phthalocyanine is activated and, consequently, the substrate is bleached. Preferred zinc phthalocyanine and a photoactivated bleaching process are described in US 4,033,718. Typically, detergent composition will contain about 0.025% to about 1.25%, by weight, of sulfonated zinc phthalocyanine.

Bleaching agents may also comprise a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", Nature 369, 1994, pp. 637-639.

Suds suppressors:

Another optional ingredient is a suds suppressor, exemplified by silicones, and silica-silicone mixtures. Silicones can generally be represented by alkylated polysiloxane materials, while silica is normally used in finely divided forms exemplified by silica aerogels and xerogels and hydrophobic silicas of various types. Theses materials can be incorporated as particulates, in which the suds suppressor is advantageously releasably incorporated in a water-soluble or waterdispersible, substantially non surface-active detergent impermeable carrier. Alternatively the suds suppressor can be dissolved or dispersed in a liquid carrier and applied by spraying on to one or more of the other components.

A preferred silicone suds controlling agent is disclosed in US 3,933,672. Other particularly useful suds suppressors are the self-emulsifying silicone suds suppressors, described in German Patent Application DTOS 2,646,126. An example of such a compound is DC-544, commercially available form Dow Corning, which is a siloxane-glycol copolymer. Especially preferred suds controlling agent are the suds suppressor system comprising a mixture of silicone oils and 2-alkyl-alkanols. Suitable 2-alkyl-alkanols are 2-butyl-octanol which are commercially available under the trade name Isofol 12 R.

Such suds suppressor system are described in European Patent Application EP 0 593 841.

Especially preferred silicone suds controlling agents are described in European Patent Application No. 92201649.8. Said compositions can comprise a silicone/ silica mixture in combination with fumed nonporous silica such as Aerosil^{R}.

The suds suppressors described above are normally employed at levels of from 0.001% to 2% by weight of the composition, preferably from 0.01% to 1% by weight.

Other components:

Other components used in detergent compositions may be employed such as soil-suspending agents, soil-releasing agents, optical brighteners, abrasives, bactericides, tarnish inhibitors, coloring agents, and/or encapsulated or nonencapsulated perfumes.

Especially suitable encapsulating materials are water soluble capsules which consist of a matrix of polysaccharide and polyhydroxy compounds such as described in GB 1,464,616.

Other suitable water soluble encapsulating materials comprise dextrins derived from ungelatinized starch acid esters of substituted dicarboxylic acids such as described in US 3,455,838. These acid-ester dextrins are, preferably, prepared from such starches as waxy maize, waxy sorghum, sago, tapioca and potato. Suitable examples of said encapsulation materials include N-Lok manufactured by National Starch. The N-Lok encapsulating material consists of a modified maize starch and glucose. The starch is modified by adding monofunctional substituted groups such as octenyl succinic acid anhydride.

Antiredeposition and soil suspension agents suitable herein include cellulose derivatives such as methylcellulose, carboxymethylcellulose and hydroxyethylcellulose, and homo- or co-polymeric polycarboxylic acids or their salts. Polymers of this type include the polyacrylates and maleic anhydride-acrylic acid copolymers previously mentioned as builders, as well as copolymers of maleic anhydride with ethylene, methylvinyl ether or methacrylic acid, the maleic anhydride constituting at least 20 mole percent of the copolymer. These materials are normally used at levels of from 0.5% to 10% by weight, more preferably form 0.75% to 8%, most preferably from 1% to 6% by weight of the composition.

Preferred optical brighteners are anionic in character, examples of which are disodium 4,4'-bis-(2-diethanolamino-4-anilino -s- triazin-6-ylamino)stilbene-2:2' disulphonate, disodium 4, - 4'-bis-(2-morpholino-4-anilino-s-triazin-6-ylamino-stilbene-2:2' - disulphonate, disodium 4,4' - bis-(2,4-dianilino-s-triazin-6-ylamino)stilbene-2:2' - disulphonate, monosodium 4',4'' - bis-(2,4-dianilino-s-tri-azin-6 ylamino)stilbene-2-sulphonate, disodium 4,4' -bis-(2-anilino-4-(N-methyl-N-2-hydroxyethylamino)-s-triazin-6-ylamino)stilbene-2,2' - disulphonate, di-sodium 4,4' -bis-(4-phenyl-2,1,3-triazol-2-yl)-stilbene-2,2' disulphonate, di-sodium 4,4'bis(2-anilino-4-(1-methyl-2-hydroxyethylamino)-s-triazin-6-ylami-no)stilbene-2,2'disulphonate, sodium 2(stilbyl-4''-(naphtho-1',2':4,5)-1,2,3, - triazole-2''-sulphonate and 4,4'-bis(2-sulphostyryl)biphenyl.

Other useful polymeric materials are the polyethylene glycols, particularly those of molecular weight 1000-10000, more particularly 2000 to 8000 and most preferably about 4000. These are used at levels of from 0.20% to 5% more preferably from 0.25% to 2.5% by weight. These polymers and the previously mentioned homo- or co-polymeric poly-carboxylate salts are valuable for improving whiteness maintenance, fabric ash deposition, and cleaning performance on clay, proteinaceous and oxidizable soils in the presence of transition metal impurities.

Soil release agents useful in compositions of the present invention are conventionally copolymers or terpolymers of terephthalic acid with ethylene glycol and/or propylene glycol units in various arrangements. Examples of such polymers are dis-

closed in US 4,116,885 and 4,711,730 and EP 0 272 033. A particular preferred polymer in accordance with EP 0 272 033 has the formula:

$$(CH_3(PEG)_{43})_{0.75}(POH)_{0.25}[T\text{-}PO)_{2.8}(T\text{-}PEG)_{0.4}]T(POH)_{0.25}((PEG)_{43}CH_3)_{0.75}$$

where PEG is $-(OC_2H_4)0-$, PO is (OC_3H_6O) and T is $(pOOC_6H_4CO)$.

Also very useful are modified polyesters as random copolymers of dimethyl terephthalate, dimethyl sulfoisophthalate, ethylene glycol and 1,2-propanediol, the end groups consisting primarily of sulphobenzoate and secondarily of mono esters of ethylene glycol and/or 1,2-propanediol. The target is to obtain a polymer capped at both end by sulphobenzoate groups, "primarily", in the present context most of said copolymers herein will be endcapped by sulphobenzoate groups. However, some copolymers will be less than fully capped, and therefore their end groups may consist of monoester of ethylene glycol and/or 1,2-propanediol, thereof consist "secondarily" of such species.

The selected polyesters herein contain about 46% by weight of dimethyl terephthalic acid, about 16% by weight of 1,2-propanediol, about 10% by weight ethylene glycol, about 13% by weight of dimethyl sulfobenzoic acid and about 15% by weight of sulfoisophthalic acid, and have a molecular weight of about 3.000. The polyesters and their method of preparation are described in detail in EP 311 342.

Softening agents: Fabric softening agents can also be incorporated into laundry detergent compositions in accordance with the present invention. These agents may be inorganic or organic in type. Inorganic softening agents are exemplified by the smectite clays disclosed in GB-A-1 400898 and in US 5,019,292. Organic fabric softening agents include the water insoluble tertiary amines as disclosed in GB-A1 514 276 and EP 0 011 340 and their combination with mono C_{12}-C_{14} quaternary ammonium salts are disclosed in EP-B-0 026 528 and di-long-chain amides as disclosed in EP 0 242 919. Other useful organic ingredients of fabric softening systems include high molecular weight polyethylene oxide materials as disclosed in EP 0 299 575 and 0 313 146.

Levels of smectite clay are normally in the range from 5% to 15%, more preferably from 8% to 12% by weight, with the material being added as a dry mixed component to the remainder of the formulation. Organic fabric softening agents such as the water-insoluble tertiary amines or dilong chain amide materials are incorporated at levels of from 0.5% to 5% by weight, normally from 1% to 3% by weight whilst the high molecular weight polyethylene oxide materials and the water soluble cationic materials are added at levels of from 0.1% to 2%, normally from 0.15% to 1.5% by weight. These materials are normally added to the spray dried portion of the composition, although in some instances it may be more convenient to add them as a dry mixed particulate, or spray them as molten liquid on to other solid components of the composition.

Polymeric dye-transfer inhibiting agents: The detergent compositions according to the present invention may also comprise from 0.001% to 10%, preferably from 0.01% to 2%, more preferably form 0.05% to 1% by weight of polymeric dye- transfer inhibiting agents. Said polymeric dye-transfer inhibiting agents are normally incorporated into detergent compositions in order to inhibit the transfer of dyes from colored fabrics onto fabrics washed therewith. These polymers have the ability of complexing or adsorbing the fugitive dyes washed out of dyed fabrics before the dyes have the opportunity to become attached to other articles in the wash.

Especially suitable polymeric dye-transfer inhibiting agents are polyamine N-oxide polymers, copolymers of N-vinyl-pyrrolidone and N-vinylimidazole, polyvinyl pyrrolidone polymers, polyvinyl oxazolidones and polyvinylimidazoles or mixtures thereof.

Addition of such polymers also enhances the performance of the enzymes according the invention.

The detergent composition according to the invention can be in liquid, paste, gels, bars or granular forms.

Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethylene glycol, PEG) with mean molecular weights of 1000 to 20000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in GB 1483591.

Granular compositions according to the present invention can also be in "compact form", i.e. they may have a relatively higher density than conventional granular detergents, i.e. form 550 to 950 g/l; in such case, the granular detergent compositions according to the present invention will contain a lower amount of "Inorganic filler salt", compared to conventional granular detergents; typical filler salts are alkaline earth metal salts of sulfates and chlorides, typically sodium sulphate; "Compact" detergent typically comprise not more than 10% filler salt. The liquid compositions according to the present invention can also be in "concentrated form", in such case, the liquid detergent compositions according to the present invention will contain a lower amount of water, compared to conventional liquid detergents. Typically, the water content of the concentrated liquid detergent is less than 30%, more preferably less than 20%, most preferably less than 10% by weight of the detergent compositions.

The compositions of the invention may for example, be formulated as hand and machine laundry detergent compositions including laundry additive compositions and compositions suitable for use in the pretreatment of stained fabrics, rinse added fabric softener compositions, and compositions for use in general household hard surface cleaning operations and dishwashing operations.

The following examples are meant to exemplify compositions for the present invention, but are not necessarily meant to limit or otherwise define the scope of the invention.

In the detergent compositions, the abbreviated component identifications have the following meanings:

LAS:	Sodium linear C_{12} alkyl benzene sulphonate
TAS:	Sodium tallow alkyl sulphate
XYAS:	Sodium C_{1X} - C_{1Y} alkyl sulfate
SS:	Secondary soap surfactant of formula 2-butyl octanoic acid
25EY:	A C_{12} - C_{15} predominantly linear primary alcohol condensed with an average of Y moles of ethylene oxide
45EY:	A C_{14} - C_{15} predominantly linear primary alcohol condensed with an average of Y moles of ethylene oxide
XYEZS:	C_{1X} - C_{1Y} sodium alkyl sulfate condensed with an average of Z moles of ethylene oxide per mole
Nonionic:	C_{13} - C_{15} mixed ethoxylated/propoxylated fatty alcohol with an average degree of ethoxylation of 3.8 and an average degree of propoxylation of 4.5 sold under the trade name Plurafax LF404 by BASF Gmbh
CFAA:	C_{12} - C_{14} alkyl N-methyl glucamide
TFAA:	C_{16} - C_{18} alkyl N-methyl glucamide
Silicate:	Amorphous Sodium Silicate (SiO_2:Na_2O ratio = 2.0)
NaSKS-6:	Crystalline layered silicate of formula δ-$Na_2Si_2O_5$
Carbonate:	Anhydrous sodium carbonate
Phosphate:	Sodium tripolyphosphate
MA/AA:	Copolymer of 1:4 maleic/acrylic acid, average molecular weight about 80,000
Polyacrylate:	Polyacrylate homopolymer with an average molecular weight of 8,000 sold under the trade name PA30 by BASF Gmbh
Zeolite A:	Hydrated Sodium Aluminosilicate of formula $Na_{12}(AlO_2SiO_2)_{12}$. $27H_2O$ having a primary particle size in the range from 1 to 10 micrometers

Citrate:	Tri-sodium citrate dihydrate
Citric:	Citric Acid
Perborate:	Anhydrous sodium perborate monohydrate bleach, empirical formula $NaBO_2.H_2O_2$
PB4:	Anhydrous sodium perborate tetrahydrate
Percarbonate:	Anhydrous sodium percarbonate bleach of empirical formula $2Na_2CO_3.3H_2O_2$
TAED:	Tetraacetyl ethylene diamine
CMC:	Sodium carboxymethyl cellulose
DETPMP:	Diethylene triamine penta (methylene phosphonic acid), marketed by Monsanto under the Trade name Dequest 2060
PVP:	Polyvinylpyrrolidone polymer
EDDS:	Ethylenediamine-N, N'-disuccinic acid, [S,S] isomer in the form of the sodium salt
Suds Suppressor:	25% paraffin wax Mpt 50°C, 17% hydrophobic silica, 58% paraffin oil
Granular Suds suppressor:	12% Silicone/silica, 18% stearyl alcohol, 70% starch in granular form
Sulphate:	Anhydrous sodium sulphate
HMWPEO:	High molecular weight polyethylene oxide
TAE 25:	Tallow alcohol ethoxylate (25)

Detergent Example I

A granular fabric cleaning composition in accordance with the invention may be prepared as follows:

Sodium linear C_{12} alkyl benzene sulfonate	6.5
Sodium sulfate	15.0
Zeolite A	26.0
Sodium nitrilotriacetate	5.0
Enzyme of the invention	0.1
PVP	0.5
TAED	3.0

Boric acid	4.0
Perborate	18.0
Phenol sulphonate	0.1
Minors	Up to 100

Detergent Example II

A compact granular fabric cleaning composition (density 800 g/l) in accord with the invention may be prepared as follows:

45AS	8.0
25E3S	2.0
25E5	3.0
25E3	3.0
TFAA	2.5
Zeolite A	17.0
NaSKS-6	12.0
Citric acid	3.0
Carbonate	7.0
MA/AA	5.0
CMC	0.4
Enzyme of the invention	0.1
TAED	6.0
Percarbonate	22.0
EDDS	0.3
Granular suds suppressor	3.5
water/minors	Up to 100%

Detergent Example III

Granular fabric cleaning compositions in accordance with the invention which are especially useful in the laundering of colored fabrics were prepared as follows:

LAS		
TAS	2.4	-
TFAA	-	4.0
45AS	3.1	10.0
45E7	4.0	-
25E3S	-	3.0

68E11	1.8	-
25E5	-	8.0
Citrate	15.0	7.0
Carbonate	-	10
Citric acid	2.5	3.0
Zeolite A	32.1	25.0
Na-SKS-6	-	9.0
MA/AA	5.0	5.0
DETPMP	0.2	0.8
Enzyme of the invention	0.10	0.05
Silicate	2.5	-
Sulphate	5.2	3.0
PVP	0.5	-
Poly (4-vinylpyridine)-N-Oxide/copolymer of vinyl-imidazole and vinyl-pyrrolidone	-	0.2
Perborate	1.0	-
Phenol sulfonate	0.2	-
Water/Minors	Up to 100%	

Detergent Example IV

Granular fabric cleaning compositions in accordance with the invention which provide "Softening through the wash" capability may be prepared as follows:

45AS	-	10.0
LAS	7.6	-
68AS	1.3	-
45E7	4.0	-
25E3	-	5.0
Coco-alkyl-dimethyl hydroxy- ethyl ammonium chloride	1.4	1.0
Citrate	5.0	3.0
Na-SKS-6	-	11.0
Zeolite A	15.0	15.0
MA/AA	4.0	4.0
DETPMP	0.4	0.4
Perborate	15.0	-

Percarbonate	-	15.0
TAED	5.0	5.0
Smectite clay	10.0	10.0
HMWPEO	-	0.1
Enzyme of the invention	0.10	0.05
Silicate	3.0	5.0
Carbonate	10.0	10.0
Granular suds suppressor	1.0	4.0
CMC	0.2	0.1
Water/Minors	Up to 100%	

Detergent Example V

Heavy duty liquid fabric cleaning compositions in accordance with the invention may be prepared as follows:

	I	II
LAS acid form	-	25.0
Citric acid	5.0	2.0
25AS acid form	8.0	-
25AE2S acid form	3.0	-
25AE7	8.0	-
CFAA	5	-
DETPMP	1.0	1.0
Fatty acid	8	-
Oleic acid	-	1.0
Ethanol	4.0	6.0
Propanediol	2.0	6.0
Enzyme of the invention	0.10	0.05
Coco-alkyl dimethyl	-	3.0
hydroxy ethyl ammonium		
chloride		
Smectite clay	-	5.0
PVP	2.0	-
Water / Minors	Up to 100%	

MATERIALS AND METHODS

Lipase activity (LU)

A substrate for lipase was prepared by emulsifying glycerin tributyrate (MERCK) using gum Arabic as emulsifier. Lipase activity is assayed at pH 7 using pH stat method. One unit of lipase activity (LU) is defined as the amount needed to liberate one micromole fatty acid per minute.

Strains and plasmids:

E. coli DH10B and DH12S are available from Gibco.

E. coli W3110 $laqI^q$

Parent *Humicola lanuginosa* lipase expression vector. The construction of the vector and its expression in *E. coli* are described in Example 4 of WO 97/04079.

Pseudomonas mendocina SD702 is described in JP-A 6-38746 and the equivalent US 5,454,971.

Pseudomonas mendocina. LDM1 is a lipase deficient derivative of *P. mendocina* SD702, prepared as follows. The 800 bp region upstream from the SD702 lipase gene (region A) was obtained by a PCR amplification using the primers IN1 (SEQ ID NO: 19) and IN2 (SEQ ID NO: 20). The 1200 bp region downstream of the SD702 lim gene (region B) was also PCR amplified using the primers IN3 (SEQ ID NO: 21) and IN4 (SEQ ID NO: 22). These two fragments were cloned next to each other and introduced into the pMFY42 plasmid. The resulting plasmid was transformed into the SD702 strain and selected for by tetracyclin. Due to the homology with either region A or B the plasmid was expected to recombine into the SD702 strains chromosome at one of these two locations. When integrated into the chromosome the plasmid may recombine out again either by leaving the same configuration as in the wt SD702 strain or by deleting the lipase and lim gene in the SD702 strain. The last case was screened for by loss of both tetracyclin resistance and lipase activity. Four strains was obtained with loss of both the tetracyclin resistance and lipase activity and one of them named LDM1. It was confirmed by PCR that the strain had lost both the lipase and lim gene.

E. coli DH12S is available from Gibco.

E. coli JM101 is available from TAKARA.

pYellow002 was prepared as follows: pJSO215 (described in Example 5 of WO 97/04079) was digested with the restriction enzymes HindIII and SphI and after Spin400 (Clontech) purification and blunt ending with Klenow polymerase, the vector was ligated to an XbaI non-phosphorylated linker (CTCTAGAG - New England Biolabs) and transformed into DH10B E. coli competent cells. The resulting plasmid was named pYellow002 and is shown in Fig. 1.

pNOVO LIP/LIM is shown in Fig. 2. It was prepared by inserting a synthetic gene called "lip/lim" into a modified T7 vector where restriction sites have been removed and other sequences removed. The modified T7 vector has Xba I and Bam HI sites at the beginning, and the Xba I site is removed by oligo insertion when introducing the gene. The synthetic gene is shown in SEQ ID NO: 1; a restriction map of the gene is shown in Fig. 3. The gene encodes the lipase from *Ps. Pseudoalcaligenes* strain M1 described in EP 334,462 and a lipase modulating factor ("lim").

pNOVO NORDISK PSD is shown in Fig. 4. It was prepared in the same manner as pNOVO LIP/LIM using the synthetic gene shown in SEQ ID NO: 2. The gene encodes LIPOSAM, a lipase derived from *Pseudomonas sp.*. SD705 (FERM BP-4772) as described in EP 721 981 and WO 96/27002. A restriction map of the gene is shown in Fig. 5.

pWYLM is a *E. coli* lipase expression plasmid shown in Fig. 6. It is constructed from pYellow 002 by replacing the *Nhe I/Bsp* EI fragment containing the LIPOLASE gene with the synthetic lip/lim gene.

pUC119 is available from Takara.

pTrc99A is available from Pharmacia.

pHSG397 is available from Takara.

pBluescriptII SK$^-$ is available from Stratagene.

pUC119Sac2.3, including 2.3kbp of *Sac* I fragment containing *P. mendocina* SD702 lipase gene, is described in JP7143883.

pS1S, including Liposam gene and Liposam lim gene is described in JP-A 8-228778.

pUC119SDL195+SDL451 is constructed from pS1S and pUC119Sac2.3 as shown in Fig. 7.

pMFY42 is a broad host range plasmid constructed from pMFY40 (Fukuda, M. and Yano, K (1985) Agric. Biol. Chem. 49 (9), 2719-2724) by replacing the Ampicillin resistance gene with a kanamycin resistance gene as described by Fukuda (1990), Iden, 44 (11), 53-58.

pMES6 is constructed from pMFY42 and pUC119SDL195+SDL451.

pTRBEN2 is constructed from pTrc99A and pUC119SDL195+SDL451 derivative. (See FIG. 9)

BEN2EBpBSII is a derivative of pBluescriptII SK$^-$, in which pTRBEN2 *EcoR* I-*Bam* HI fragment is inserted . (see FIG. 8)

pHSG397pp is a pHSG397 derivative. pHSG397 has been digested with *Pvu* I, blunted by T4 polymerase, and self-ligated. (See FIG.10)

pHSG397ppBEN2 is a derivative of pHSG397pp, in which pTRBEN2 *Eco* RI-*Bam* HI fragment is inserted . (See FIG.10)

36+83pUC, containing SD702 lipase promoter region and the cloning sites for *Nhe* I-*Bam* HI fragment. (See FIG. 10)

LMNBpUC is constructed from pUC119SDL195+SDL451 and 36+83pUC. (See FIG. 10)

Primers

Primer 27: SEQ ID NO: 3
Primer 36: SEQ ID NO: 4
Primer 40: SEQ ID NO: 5
Primer 43: SEQ ID NO: 6
Primer XHO-A: SEQ ID NO: 7
Primer HPA-A: SEQ ID NO: 8
Primer HPA-S: SEQ ID NO: 9
Primer D229G+230F: SEQ ID NO: 10
Primer D250N: SEQ ID NO: 11
Primer D272S: SEQ ID NO: 12
Primer 101: SEQ ID NO: 13
Primer 102: SEQ ID NO: 14
Primer 104: SEQ ID NO: 15
Primer 105: SEQ ID NO: 16
Primer 106: SEQ ID NO: 17
Primer 127: SEQ ID NO: 18

Medium and substrates

LB medium

10g/L	tryptone
5g/L	yeast extract
5g/L	NaCl
	pH 7.2

C9 medium

4g/L	$(NH_4)_2SO_4$	
10g/L	K_2HPO_4	
4g/L	KH_2PO_4	
3g/L	$MgSO_4.\cdot 7H_2O$	(sterilize independently before mixing)
0.5g/L	$CaCl_2\cdot$2H2O	(sterilize independently before mixing)
3g/L	Na_2CO_3	(sterilize independently before mixing)
20g/L	Tween80	(sterilize independently before mixing)
0.3g/L	$FeSO_4\cdot 7H_2O$	(sterilize by filtration)

0. 05g/L	$MnSO_4 \cdot nH_2O$	(sterilize by filtration)

Additives Antibiotic and galactosides (If required)
Ampicillin to 250µg/ml
Kanamycin to 20µg/ml
IPTG to 0.4%

Olive oil assay plate Olive oil emulsion (2% P.V.A.: Olive oil = 2:1),
Brilliant green (Indicator, 0.004%),
50mM carbonate buffer, pH10
EGTA (final concentration, 5mM)
PCS solution (final concentration, 0 to 0.5g/L)

8g/L PCS solution:

0.48g	DOBANOL 25-3
0.46g	DOBANOL 25-7
0.42g	SDS
1.2g	$NaBO_3 \cdot H_2O$

Carbonate buffer, adjust to 1L

Methods

Transformation of *E. coli*

E. coli transformation for constructing libraries and subcloning are carried out by electroporation (BIO-RAD Gene Pulser).

Cultivation of transformants

E. coli transformants are cultivated in LB medium with appropriate antibiotics.

Preparation of plasmid DNA

Plasmid DNAs are prepared by alkaline method (Molecular Cloning, Cold Spring Harbor) or with Qiagen® Plasmid Kit.

Recovery of DNA fragments

DNA fragments are recovered from agarose gel by SuprecTM-01(TAKARA) or agarase (NEB).

PCR

PCR is carried out by PTC-200 DNA Engine.

First screening assay method for *E. coli* mutagenized libraries

1) Provide LB containing ampicillin plates with a filter (Cellulose acetate) on the top.

2) Spread *E. coli* cells containing a parent *Ps. pseudoalcaligenes* lipase and lim gene or a mutated *Ps. pseudoalcaligenes* lipase and lim gene in question on the filter and incubate overnight at 37°C.

3) Transfer the colonies on the filter to a new LB plate containing ampicillin and IPTG and incubate overnight at 27°C.

4) Transfer the colonies on the filter to an olive oil assay plate and incubate overnight at 27°C.

5) Identify colonies found in step 4) having blue halos

Determination of DNA sequences

ABI PRISMTM 310 Genetic Analyzer is used for determination of all the DNA sequences.

***Pseudomonas* transformation**

1) Inoculate *Pseudomonas* host, LDM1, in 5ml of LB medium and cultivate at 37°C overnight.

2) Inoculate 0.1ml of the seed culture in fresh 10ml of LB medium

3) Cultivated at 37°C until the OD_{680} reaches 0.7.

4) Centrifuge the culture and wash the cells twice with distilled water.

5) Re-suspend the cell in 0.5ml of distilled water.

6) Add 5µg of DNA solution to the cell suspension.

7) Transfer the cell suspension into a gene pulser cuvette (0.4cm gap).

8) Set electroporation apparatus to 25 µF, 1000 Ω, 2.5 kV.

9) Put the cuvette in GENE PULSER (BIO-RAD) and pulse once.

10)Transfer the cell suspension into a 15ml falcon tube and diluted with 2ml of LB medium.

11)Cultivate at 37°C for 60min.

12)Spread the cells to L8 medium containing 20ppm of kanamycin and incubate at 37°C for 1 or 2 days.

Plate assay for *Pseudomonas* lipolytic enzyme transformants

Pseudomonas transformants are streaked on cellulose acetate membrane on LB plate containing 20 ppm of kanamycin and grown at 37°C overnight. The membrane with colonies are transferred onto LB plate containing olive oil and brilliant green and incubated at 37°C for over 6 hours.

Cultivation of *Pseudomonas*

Pseudomonas transformants and the host strain are cultivated in 1ml of C9 medium with/without 20ppm of Kanamycin at 37°C for over 16 hours with shaking for small scale fermentation in tube.

Fermentation of *Pseudomonas*

Fed batch fermentation of *Pseudomonas* in a small tank is carried out with Tween80, rape seed oil or oleic acid as a carbon source and ammonium sulfate as a nitrogen source. A shake flask culture of *Pseudomonas* strain in question is inoculated into a medium comprising 0.5% of the carbon source and 0.2% of the nitrogen source. After 10 hours of cultivation at pH 8.0 and 30°C, continuous supply of carbon source is initiated, keeping pH at 8.0 by supplying 12.5% of ammonia water and excess oxygen supply.

The cultivation is continued for 3 days, when lipolytic enzyme is recovered by centrifugation, filtration and acetone precipitation. Further purification may be carried out by hydrophobic chromatography.

Purification of *Pseudomonas* lipolytic enzyme variants

1) Centrifuge fermentation broth and discard cell debris.
2) Filter the supernatant through a 0.45μ filter.
3) Precipitate enzyme in this filtrate with 50% acetone.
4) Dissolve the precipitate in water and was add solid ammonium sulfate to the concentration of 20%.
5) Pack a 50ml column with TSK gel Phenyl-Toyopearl 650.
6) Washed the column and equilibrate with 20% saturated ammonium sulfate.
7) Apply the enzyme solution into a Phenyl Toyopearl column.
8) Wash the column with 20% saturated ammonium sulfate until all unbounded materials were washed out.
9) Elute the column with water.
10) Pool the fractions containing lipase activity

Detergent filter assay

<u>Procedure</u>

1) Provide LB agar plates (with 50-100 ug/ml ampicilin) with a low protein binding filter (Cellulose acetate) and spread E. coli cells containing the parent lipase gene or the mutated lipase gene in question on the double filter and incubate for 1day at 37°C.

2) Transfer the filter to a new LB agar plate (with 50-100 ug/ml ampicilin and 1 mg IPTG) which has been provided with a protein binding filter (Nylon membrane) and incubate for 4-8 hours at 30°C.

3) Keep the colonies on the top filter by transferring the top filter to a new plate.

4) Remove the protein binding filter to an empty petri dish.

5) Pour an agarose solution comprising an olive oil emulsion (2% P.V.A.: Olive oil=3:1), Brilliant green (indicator,0.004%), 100 mM tris buffer pH9, PCS detergent (see composition below) and EGTA (final concentration 5 mM) on the bottom filter so as to identify colonies expressing lipolytic activity in the form of blue-green spots.

6) Identify colonies found in step 5) having a better performance in detergent as compared to the parent lipase.

<u>**An alternative screening assay is the following:**</u>

Transfer the protein binding filter from step 4 to an agarose plate comprising an olive oil emulsion (2% P.V.A.: Olive oil=2:1), Brilliant green (indicator,0.004%), 100 mM tris buffer pH 9 or 10 and the detergent or detergent component, e.g. PCS-plates. The protein binding filter should have the colony side facing the screening plate. Identify colonies expressing lipase activity in the form of blue-green spots.

Alternatively, the non-protein binding filter (or a protein binding filter) carrying the *E. coli* colonies may be used directly on the screening plate.

Construction of random mutagenized libraries

<u>**Rationale and mathematics behind the design of random mutagenized libraries**</u>

The overall rationale for the random mutagenesis is to mimic the evolution in nature where a low continuous mutagenesis is coupled to a continuous selection for a better mutant which is then further mutagenized. Similarly, the recent in vitro evolution studies described in the literature have been performed with consecutive rounds of mutagenesis with increasing selection pressure (for a review see Joyce 1992). We are adapted this by using the wt gene in the first rounds of mutagenesis. Improved variants are then used in the next rounds of mutagenesis (to improve by small steps). Screening is performed under wash correlated conditions that are only just enough to knock out the wt enzyme activity or improved variants activity. This means that we increase the stringency of screening when better and better variants are isolated.

To increase the number of exchanges and to increase the likelihood of finding improved variants, localized random mutagenesis are performed. Important regions deduced from the structure of the *Ps. pseudoalcaligenes* lipase may be selected.

b) Random mutagenesis of an entire lipolytic enzyme coding gene

A plasmid containing the gene to be mutated is treated with 12 M formic acid for 20 min. at room temperature. The resulting mutated lipase gene is amplified from the formic acid treated plasmid using PCR under mutagenic conditions (0.5 mM $MnCl_2$ and 1/5 the normal amount of ATP, see e.g. Leung et al., 1989. This treatment is expected to give a broad range of mutations since formic acid gives mainly transversions and PCR generated mutations mainly transitions.

The resulting PCR fragments are cloned either by double recombination (Muhlrad et al., 1992) in vivo into the shuttle vector or digestion and ligation into the shuttle vector and transformation of *E. coli.*

Localized random mutagenesis

A mutagenic primer (oligonucleotide) is synthesized which corresponds to the part of the DNA sequence to be mutagenized except for the nucleotide(s) corresponding to amino acid codon(s) to be mutagenized. Subsequently, the resulting mutagenic primer is used in a PCR reaction with a suitable opposite primer. The resulting PCR fragment is purified and digested and cloned into the shuttle vector. Alternatively and if necessary, the resulting PCR fragment is used in a second PCR reaction as a primer with a second suitable opposite primer so as to allow digestion and cloning of the mutagenized region into the shuttle vector. The PCR reactions are performed under normal conditions.

When synthesizing the oligonucleotides used for the localized random mutagenesis, calculation of the doping level is important to estimate the mutagenesis frequency. The frequency of nucleotide exchanges can be calculated using the Binomial distribution formula:

$$P(i) = \frac{N!}{i!(N-i)!} \times p^i \times (1-p)^{N-i}$$

Where N = the number of doped oligo nucleotides; p = the fraction of none wt nucleotides; i = number of nucleotide exchanges; P(i) = the probability for the i number of exchanges. It is difficult to calculate the exact number of aa exchanges from the number of nucleotide exchanges, because the third position in a codon for most of the aa can be two or all four nucleotides with out changing the aa. The same is the case for the first or second position for the three aa with 6 codons. For estimating the number of aa exchanges a Monte-Carlo simulation is more appropriate. For example the program called RAMHA performs such a simulation (described in Siderovski and Mak 1993). This program simulates the synthesis of e.g. 10,000 oligonucleotides with the desired doping and calculates the frequency of 0 to n aa exchanges.

A doping example

The relationship between doping and aa exchanges in a region of 13 codons is (calculated using a Monte Carlo simulation):

Percent doping level	0 mutations	1	2	3	4	5	6	7
5%	0.2	0.35	0.27	0.13	0.05	0	0	0
10%	0.04	0.13	0.24	0.25	0.19	0.11	0.05	0
15%	0.005	0.03	0.10	0.20	0.24	0.23	0.13	0.07

The possible number of combinations of aa exchanges for 13 aa can be calculated using the formula:

$$N = \frac{y!}{x!(y-x)!} 20^x$$

y = number of aa mutagenized
x = number of aa exchanges

1 aa exchange in 13 aa	= 260 possible exchanges	
2 - - - -	= 31200	- combinations
3 - - - -	= 2.3×10^6 -	-

From this follows that when screening e.g. 100,000 colonies of a library doped with 10% in 13 codons giving the distribution shown in the above table will mean screening of about 13,000 with one aa exchange (13%). There are, however, only 260 possible one aa exchanges, so a large number of the same one aa exchanges are being screened. A higher doping of e.g. 15% (in the above table) will give fewer one aa exchanges (about 3%), however, the two aa exchanges will also be lowered to a degree (10%) that will not enable screening of the 31200 possible combinations with a screenings capacity around 100.000.

Finally, the aa exchanges are biased by the origin of the wt amino acid. E.g. it takes only one nucleotide exchange to change Glu to Ala, but three from Glu to Phe. This means that the probability is lower for the aa exchanges that requires 2 or 3 nucleotide exchanges than for those that requires one nucleotide exchange. Therefore we have in some cases allowed more than one aa at positions where we know it is possible. We have always chosen G/C at the third position of the codons with four or six codons. This lowers the bias of the wt codon and also lowers the likelihood of stop

codon (from 4.7% to 3.1% if completely scrambled). For a calculation of the probability of whether a given pool size contain the most probable and least probable replacement mutants, see Palzkill et al. 1994.

Anti-termination strategies

In order to avoid premature truncated proteins nonsense mutations should be avoided in the codons with a potential to form stop codons. For codons that can be substituted with alternative codons with out the potential to form stop codons, the following strategies can be used:

Gly: GGA	GG(G,C,T)
Leu: TT(A,G)	CTN
Arg: (A,C)GA	(A,C)GG or CG(C,T)
Ser: TC(A,G)	TC(C,T) or AG(C,T)

The following aa can, however, only be specified with codons exhibiting stop-codon potential: Cys, Glu, Lys, Gln, Trp, and Tyr. Therefore only the doping can be designed to circumvent the random placement of nucleotides producing stop codons. For example:

Glu (similar for Lys and Gln): (90%G/5%C,A) (90%A/3.3%C,G,T) (90%A/3.3%C,G,T). No TAA or TAG = STOP.

Tyr (similar for Cys): (90%T/3.3%A,C,G) (90%A/3.3%C,G,T) (90%C/10%T). No TAG or TAA = STOP.

Trp: (90%T/3.3%A,C,G) (90%G/5%C,T) (90%G/5%C,T). No TGA or TAG = STOP.

Such a strategy will of course abolish certain a.a. exchanges. Using these strategies the number of premature truncated proteins will be lowered dramatically.

3-cycle wash performance

The 3-cycle wash performance of a modified lipolytic enzyme of the invention can be evaluated on the basis of the enzyme dosage in mg of protein (or LU) per liter compared to the parent lipolytic enzyme. Wash trials are carried out in 150 ml beakers placed in a thermostated water bath. The beakers are stirred with triangular magnetic rods.

The experimental conditions are as follows:

Method:	3 cycles with overnight drying between each cycle
Wash liquor:	100 ml per beaker
Swatches:	6 swatches (3.5 x 3.5 cm, stained with lard colored with 0.75 mg Sudan red/gram of lard) per beaker
Detergent:	Detergent I, pH adjusted to 10.2

Enzyme conc.:	0.075, 0.188, 0.375, 0.75 and 2.5 mg of lipase protein per liter
Time:	20 minutes
Temperature:	30°C
Rinse:	15 minutes in running tap water
Drying:	overnight at room temperature (~20°C, 30-50% RH)
Evaluation:	after the 3rd wash, the reflectance at 460 nm was measured.

Evaluations of wash results

Dose-response curves are compared for the modified lipolytic enzyme and the parent lipolytic enzyme. The dose-response curves is calculated by fitting the measured data to the following equation:

$$DR = DR_{max} \frac{C^{0.5}}{K + C^{0.5}} \qquad \text{(I)}$$

where DR is the effect expressed in reflectance units

C is the enzyme concentration (mg/l)

DR_{max} is a constant expressing the maximum effect

K is a constant; K^2 expresses the enzyme concentration at which half of the maximum effect is obtained.

Based on the characteristic constants DR_{max} and K found for each modified lipolytic enzyme as well as the parent lipolytic enzyme, improvement factors are calculated. The improvement factor, defined as

$$f_{improve} = C_{parent}/C \quad \text{(II)}$$

expresses the amount of modified lipase protein needed to obtain the same effect as that obtained with 0.25 mg/l of the reference parent protein (C_{parent}).

Thus, the procedure for calculating the improvement factor is as follows:

1) The effect of the parent protein at 0.25 mg/l (DR_{parent}) was calculated by means of equation (I);

2) the concentration of the modified lipolytic enzyme resulting in the same effect as the parent enzyme at 0.25 mg/l was calculated by means of the following equation:

$$C = (K_{modify} \frac{DR_{parent}}{DR_{max(modify)} - DR_{parent}})^2 \qquad \text{(III)}$$

3) the improvement factor was calculated by means of equation (II).

Assay for test of First Wash effect

The wash performance of lipolytic enzymes was tested in a one cycle wash trial carried out in a thermostated Terg-O-Tometer (TOM) followed by linedrying.

The experimental conditions were as follows:

Wash liquor: 1000 ml per beaker

Swatches: 7 cotton swatches (9 x 9 cm) per beaker.

Stain: Lard colored with Sudan red (Sigma) (0.75 mg Sudan red/g of lard). 50 μl of lard/Sudan red heated to 70°C were applied to the center of each swatch. After application of the stain the swatches were heated in an oven for 25 minutes at 75°C. Stored overnight at room temperature prior to the first wash.

Water: 3.2 mM Ca^{2+}/Mg^{2+} (in a ratio of 5:1)

Detergent: 5 g/l of Detergent Composition A or Detergent B. pH adjusted artificially to 10 by NaOH.

__Detergent Composition A:__

0.300 g/l of alkyl sulphate (AS; C_{14-16})

0.650 g/l of alcohol ethoxylate (AEO; C_{12-14}, 6EO)

1.750 g/l of Zeolite P

0.145 g/l of Na_2CO_3

0.020 g/l of Sokalan CP5

0.050 g/l of CMC (carboxy- methyl-cellulose)

Mixed in 3.2 mM Ca^{2+}/Mg^{2+} (5:1) in Milli-Q water, pH 10.2

__Detergent Composition B__

As Detergent Composition A but additional containing the following bleaching agents:

g/l Ssodium carbonate peroxyhydrate

0.300 g/l TAED (tetra-acetyl-ethylene-diamine)

Concentration of lipolytic enzyme (in Detergent composition A as well as B): 0 and 1250 or 12500 LU/l

Wash Time: 20 minutes

Wash temperature: 30°C

Rinse: 15 minutes in running tap water

Drying: Overnight at room conditions (approx. 20°C, 30-40% RH).

Evaluation: The reflectance was measured at 460 nm. Afterwards, the fatty matter was extracted from the swatches with chloroform in a Soxhlet extraction apparatus, distilling off the solvent and determining the amount of fatty matter left on the swatches gravimetrically. The percentage residual fatty material may alternatively be

determined as a fraction of the fatty matter removed by detergent without lipolytic enzyme using thin layer chromatography(TLC)/Flame Ionization Detector (FID)].

The percentage of lard removed is determined as:

% removal defined as:

[(remaining fat on swatches washed with detergent without lipolytic enzyme) minus (remaining fat on swatches washed with detergent with lipolytic enzyme)] divided by (remaining fat on swatches washed with detergent without lipolytic enzyme) and multiplied by 100%, or

delta reflectance(ΔR) defined as:

(R(swatches washed in detergent with lipase)-R(swatches washed in detergent without lipase). The reflectance (which may also be termed remission) is measured on an Elrepho 2000 apparatus from Datacolor which illuminates the sample with 2 xenon blitzlambs and measures the amount of reflected light so that entirely white correspond to a 100% reflection and entirely black a 0% reflection.

Media and substrates:

LB-medium: 10 g Bacto-tryptone, 5 g Bacto yeast extract, 10 g NaCl in 1 liter water.

Litex Agarose HSB 2000 (CAT NO: F90472)

BG-reagent: 4 mg/ml Brilliant Green (BG) dissolved in water

Substrate 1:

10 ml Olive oil (Sigma CAT NO. 0-1500)

20 ml 2% polyvinyl alcohol (PVA)

The Substrate is homogenized for 15-20 minutes.

PCS detergent

10 g/l:

SDS	0.52 g
Dobanol 25-3	0.60 g
Dobanol 25-1	0.58 g
$NaBO_3H_2O$	1.50 g

Ad 1 liter 0.1 M Tris buffer (pH 9), and dilute further with the Tris buffer to the double concentration of the desired concentration on the PCS plates.

PCS-plates

Solution for making PCS plates

Brilliant Green (BG-reagent)	10 ml
Substrate 1	24 ml
PCS detergent	500 ml
2% agarose (in TRIS buffer (pH 9)	500 ml

Lipase Substrate (Sigma catalogue no. 800-1)
Brilliant Green (Merck, art. No. 1.01310)

EXAMPLES

EXAMPLE 1

Construction of random lipolytic enzyme variants

Random mutagenized libraries of the entire *Pseudomonas* lipolytic enzyme gene and specific regions thereof are prepared as described in Materials and Methods above.

One oligonucleotide is synthesized for each of these regions comprising e.g. 90% of the wild type nucleotides and 3.33% of each of the other three nucleotides at amino acid codons wanted to be mutagenized. Where possible without changing the amino acid, the third nucleotide (the wobble base) in codons is synthesized with 50%G/50%C to give a larger likelihood for changes to amino acids with one or two codons.

The mutagenic primers are used in a PCR reaction with a suitable opposite primer. The resulting PCR fragments are purified and digested and cloned into the expression vector. A large number of colonies are screened from the different libraries using the detergent filter assay described in Materials and Methods above.

Local random mutagenesis is also performed on two or more regions simultaneously. For instance, the following doping scheme may be used for the *Ps. pseudoalcaligenes* lipase:

Region 6-18:	6-14 90% wt/10% random
	15-18 93% wt/7% random
Region 21-39:	21-24 93% wt/7% random
	25-31 90% wt/10% random
	32-37 93% wt/7% random
	38-39 90% wt/10% random
Region 46-74:	46-74 90% wt/10% random
Region 88-100:	88-100 93% wt/7% random
Region 109-136:	109-114 93% wt/7% random
	115-136 90% wt/10% random
Region 154-180:	154-159 90% wt/10% random
	160-168 93% wt/7% random
	169-180 90% wt/10% random
Region 188-200:	188-200 90% wt/10% random
Region 222-231:	222-231 90% wt/10% random

Region 258-268: 258 and 261-268 90% wt/10% random
259 and 260 unchanged

In particular the localized random mutagenesis may be performed in one or more of the following regions:

15-18, 21-36, 110-136, 154-167, 222-231, 258-266
or in one or more of the following regions:
6-14, 37-39, 46-74, 88-100, 109, 168-180, 188-200, 267-268
preferably using the doping scheme given above.

EXAMPLE 2

The DNA sequences encoding the best performing variants obtained in Example 1 may be recombined at random using conventional recombination techniques. The resulting recombined DNA sequences are expressed and a screening for novel lipase variants with improved wash performance may be selected based on the principles disclosed in the Materials and Methods section herein. For instance, when the DNA sequences to be combined comprises homologous fragments, the combination is preferably achieved by homologous cross-over, e.g. by use of conventional methods such as US 5, 093, 257, or by gene shuffling (Stemmer (1994), Proc. Natl. Acad. Sci. USA, vol. 91, 10747-10751; Stemmer (1994), Nature, vol. 370, 389-391; Smith (1994), Nature vol 370, page 324-25), WO 95/17413. Gene shuffling means recombination of nucleotide sequence(s) between two or more homologous DNA sequences resulting in output DNA sequences having a number of nucleotides exchanged.

Of particular interest is the *in vivo* Gene Shuffling Method described in PCT DK96/00343, which is based on the following procedure:

a) forming at least one circular expression vector comprising a DNA sequence encoding a parent lipase or a substantial part thereof,

b) opening said circular expression vector within the DNA sequence encoding the lipase or part thereof,

preparing at least one DNA fragment comprising a DNA sequence homologous to at least a part of the enzyme coding region on at least one of the circular expression vector(s),

d) introducing at least one of said opened vector(s), together with at least one of said homologous DNA fragment(s) covering full-length DNA sequences encoding said lipase or a part thereof, into a recombination host cell,

e) cultivating said yeast recombination host cell under conditions conducive for recombination between the homologous DNA fragments to take place, and screening for positive lipase variants with an improved wash performance.

EXAMPLE 3

Site-directed in vitro mutagenesis of lipolytic enzyme genes

One approach which may be used for introducing mutations into the lipase gene is described in Nelson & Long, Analytical Biochemistry, 180, 147-151 (1989). It involves the 3-step generation of a PCR (polymerase chain reaction) fragment containing the desired mutation introduced by using a chemically synthesized DNA-strand as one of the primers in the PCR-reactions. The construction of a PCR fragment may be performed in accordance with methods known in the art. From the PCR generated fragment, a DNA fragment carrying the mutation can be isolated by cleavage with restriction enzymes and re-inserted into the expression plasmid.

An alternative method for the construction of variants of a *Pseudomonas* lipase involve the use of the commercial kit, Chameleon double-stranded, site-directed mutagenesis kit according to the manufacturer's instructions.

The gene encoding the lipase in question is inserted into the plasmid. In accordance with the manufacturer's instructions the ScaI site of the Ampicillin gene is changed to a MluI site. The desired mutation is introduced into the lipolytic gene in question by addition of appropriate oligos comprising the desired mutation. The PCR reactions are performed according to the manufacturer's recommendations.

<u>DNA sequencing</u> is performed by using Applied Biosystems ABI DNA sequence model 373A according to the protocol in the ABI Dye Terminator Cycle Sequencing kit.

EXAMPLE 4

Expression of *Pseudomonas* lipase variants in Pseudomonas

The lipase variants may be produced as described in Example 7 of WO 95/14783. More specifically, the gene encoding the variant is inserted into the broad host range plasmid pMFY42 which is subsequently transformed into the lipase negative *Ps. mendocina* strain LDM1. The transformed strain is cultivated in an appropriate medium and the resulting expressed lipase variant recovered from the medium.

EXAMPLE 5

Expression of *Pseudomonas* lipolytic enzyme variants in *E. coli* for screening of random mutagenized library

The genomic DNA fragment of the *Ps pseudoalcaligenes* lipase encoding fragment containing both the lipase gene and the lim gene is cloned into the *E. coli* expression vector pTrc99A purchased from Pharmacia between the Ptrc promoter and 5S terminator. The expression is induced with IPTG as described in materials and methods.

Alternatively the genomic DNA fragment of the *Ps pseudoalcaligenes* lipase encoding fragment containing both the lipase gene and the lim gene is cloned into the E. coli expression vector pSX581 by fusing the N-terminal of the mature part of the lipase gene to the Acremonium signal sequence present in pSX581 and deleting the *Humicola lanuginosa* lipase gene present in pSX581. The lim gene is located downstream and followed by the 5S terminator.

EXAMPLES 6-13

Construction and testing of mutant lipases

Ps. pseudoalcaligenes lipase mutagenized libraries of error prone PCR and localized random mutagenesis in *E. coli* were screening on olive oil assay plates described below in detail. The obtained positives in the first screening were isolated, sequenced and cloned into a *Pseudomonas* expression vector and transformed into *Pseudomonas.*

Pseudomonas expression vectors for module shift variants, site-directed mutants and N-terminal extended mutants of *Ps. pseudoalcaligenes* lipase were constructed and transformed into *Pseudomonas* without evaluating their *E. coli* transformants on olive oil assay plates.

All the *Pseudomonas* transformants were cultivated in small scale and their broths were subjected to olive oil assay plate to be selected for the next washing evaluation.

For the washing test, large scale fermentations were carried out for preparing large amount of the lipolytic enzyme samples.

EXAMPLE 6

Construction of module shift variants

<u>Construction of expression plasmids of LMV1-LMV8</u>

<u>Construction of module shifted *Ps. pseudoalcaligenes* lipase variant genes in *E. coli* (See FIG. 11 & 12).</u>

<u>1.a</u>. pNovo Nordisk-PSD was digested with 2 enzymes: *Nhe* I and *Spe* I, *Spe* I and *Sac* II, *Sac* II and *Sac* I, *Sac* I and *Kpn* I, *Kpn* I and *Nde* I and the smaller fragments among the obtained 2 fragments were isolated. pNovo Lip/Lim was also digested with the same enzymes and the larger fragments were ligated to the pNovo Nordisk-PSD fragments and transformed into *E. coli* JM101. The obtained transformants harboring the plasmids containing the genes encoding module shifted *Ps. pseudoalcaligenes* lipase variants were isolated. The module shifted plasmids were termed as follows: pNheISpeI, pSpeISacII, pSacIISacI, pSacIKpnI, pKpnINdeI

1.b. pNovo Nordisk-PSD was digested with *Nhe* I and *Eco* RV and the small fragments among the obtained 2 fragments were isolated. pNovo Lip/Lim was digested with *Sph* I, blunted with T4 DNA polymerase and digested with *Nhe* I. The larger fragment was ligated to the *Nhe* I-*Eco* RV fragment of the pNovo Nordisk-PSD and transformed into *E. coli* JM101, and the obtained transformant harboring the plasmid containing the two lipase (wild-type *Ps. pseudoalcaligenes* lipase and Liposam) genes was isolated. The plasmid was digested with *Sac* II, *Sac* I, or *Kpn* I, self-ligated and transformed into *E. coli* JM101. The resulting transformants harboring the plasmids containing module shifted *Ps. pseudoalcaligenes* lipase variant genes were isolated. Those plasmids were termed as follows: pNheISacII, pNheISacI, pNheIKpnI

Construction of plasmids containing SD702 lipase promoter region and signal sequence

The 51bp oligonucleotide consist of the native sequence of SDL-451,
5'-GGCTCCTCGAACTACACCAAGACCCAGTACCCGATCGTCCTGACC
CACGCC-3' (SEQ ID NO. 23)
was synthesized as a PCR primer. Using this primer and primer 40, a 2.0kbp fragment was amplified using pUC119SDL-195+SDL-451 as a template. This fragment was mixed with *EcoR* I-*Hind* III fragment of pUC119SDL-195+SDL-451, boiled for 5 minutes and cooled slowly till room temperature for annealing. Then *Taq* polymerase was added and incubated at 72°C for 10 minutes for elongation.

Using the following primer; 5'-GGGAATTCGAGCTCGCTCCTACACGAAG-3' (SEQ ID NO. 24), and primer 40, a 2.9kbp fragment was amplified by PCR. The fragment was digested with *Hind* III and ligated to the larger fragment of pUC119SDL-195+SDL-451 digested with *Hind* III.

A *Nhe* I site was introduced into pUC119SDL-195+SDL-451 by the similar PCR procedure described above using the following primer; 5'-GCCTGCTCGCTAGCGGCCAGA-3' (SEQ ID NO. 25).

The resulting fragment was digested with *Eco* RI and *Kpn* I and ligated to the larger fragment of pUC119SDL-195+SDL-451 digested with *Eco* RI and *Kpn* I. The resulting plasmid was termed pUC119SDL-195+SDL-451+NheI.

A *Bam* HI site in pUC119SDL-195+SDL-451+*Nhe* I was eliminated by PCR. The primer containing the disrupted BamHI site; 5'-TGTCCTGCGGGTCCAGCGACG-3' (SEQ ID NO. 26), was synthesized. With this primer and primer 36, a 2.6kbp fragment was amplified by PCR and the resulting fragment was annealed with a *Hind* III fragment of pUC119SDL-195+SDL-451+NheI. With primer 36 and the following primer containing a *Bam* HI site: 5'-GGGGATCCCCACTCCAGAATCGGTG-3' (SEQ ID NO. 27), a 2.9kbp fragment where a *Bam* HI site was introduced at 3'-terminal, was amplified,

digested with *Eco* RI and *Bam* HI and ligated to *Eco* RI and *Bam* HI site of pUC119. The resulting plasmid was termed pUC119SDL-195+SDL-451+ *Nhe* I+*Bam* HI.

pUC119SDL-195+SDL-451+ *Nhe* I+*Bam* HI was digested with *Eco* RI and *Bam* HI and ligated into *Eco* RI and *Bam* HI site of pTrc99A. The resulting plasmid was termed pTRBEN2.

pTRBEN2 was digested with *Eco* RI and *Bam* HI, and ligated into *Eco* RI and *Bam* HI site of pBluescriptIISK⁻. The resulting plasmid was termed BEN2EBpBSII.

pHSG397 was digested with *Pvu* I and subsequently the ends of the fragment were blunted with T4 DNA polymerase and self-ligated. The resulting plasmid was termed pHSG397pp.

pTRBEN2 was digested with *Eco* RI and *Bam* HI, and ligated into pHSG397pp digested with *Eco* RI and *Bam* HI. The resulting plasmid was termed pHSG397ppBEN2.

Connection of SD702 lipase promoter region, signal sequence, and *Ps. pseudoalcaligenes* lipase variant genes

3.a. pSpeIsacII, pSacIISacI, and pKpnINdeI described in 1.a. were digested with *Sph* I and *BamH* I and the smaller fragments were isolated out of two obtained fragments and ligated to the larger fragment of BEN2EBpBSII digested with *Sph* I and *BamH*. The constructed plasmids were termed as follows:pSpeISacIIBEN2, pSacIISacIBEN2, pKpnINdeIBEN2.

3.b. pNheISpeI, pSacIKpnI, pNheISacII, pNheISacI, and pNheIKpnI described in 1.b. were digested with *Pvu* I and *Bam* HI and the smaller fragments were isolated. pHSG397ppBEN2 was also digested with *Pvu* I and *Bam* HI and the obtained larger fragment was ligated to them. The constructed plasmids were termed as follows: pNheISpeIBEN2, pSacIKpnIBEN2, pNheISacIIBEN2, pNheISacIBEN2, pNheIKpnIBEN2.

Construction of pMES6

pS1S was digested with *Eco* RI and a 2.7kbp fragment was sub-cloned into pUC119 (pUC119SDL-451EcoRI). A *Eco* RI site at the 3'-terminal of sense strand of the *Eco* RI fragment was replaced with a *Hind* III site by PCR procedure.

The PCR reaction was performed with the following primer:

5'-AAAAGCTTGATCTACGCCCCAGGACC-3' (SEQ ID NO. 28), and primer 36 using pUC119SDL-451EcoRI as a template. An amplified 2.7kbp fragment was digested by *Eco* RI and *Hind* III and sub-cloned into pUC119 (pUC119SDL-451).

pUC119SDL-195 was digested with *Eco* RI and *Sph* I and the 0.9kbp of *Eco* RI-*Sph* I fragment was isolated. pUC119SDL-451 was also digested with *Eco* RI and *Sph* I

and the larger fragment corresponding to the *Pseudomonas* expression vector region was ligated to the *Eco* RI-*Sph* I fragment of pUC119SDL-195+ SDL-451.

A *Hind* III site of *Eco* RI-*Hind* III fragment of pUC119SDL-195+SDL-451 was newly made at the EcoRI site by PCR using the following primer; 5'-AAGAATTCCCACTCCAGAATCGGTG-3' (SEQ ID NO. 29)

The procedure was the same as that described above. An amplified 3.2kbp *Eco* RI fragment was ligated to EcoRI site of pMFY42. The constructed plasmids were termed pMES6.

Transfer into *Pseudomonas* expression plasmid

The constructed plasmids described in 3.a. and 3.b. were digested with *Hind* III and the smaller fragments among the resulting fragments were isolated. pMES6 was also digested with *Hind* III and the larger fragment corresponding to vector region was ligated to them and transformed into *E. coli* JM101. The obtained transformant harboring the plasmid containing the module shifted *Ps. pseudoalcaligenes* lipase variant gene was isolated. The constructed plasmids were termed as follows:

pNheISpeIBEN2 HindIII fragment was transferred into pLMV1R
pSpeISacIIBEN2 HindIII fragment was transferred into pLMV2R
pSacIISacIBEN2 HindIII fragment was transferred into pLMV3R
pSacIKpnIBEN2 HindIII fragment was transferred into pLMV4R
pKpnINdeIBEN2 HindIII fragment was transferred into pLMV5R
pNheISacIIBEN2 HindIII fragment was transferred into pLMV6R
pNheISacIBEN2 HindIII fragment was transferred into pLMV7R
pNheIKpnIBEN2 HindIII fragment was transferred into pLMV8R
(See FIG. 13)

Construction of expression plasmids of LMV5 variants

Construction of plasmids containing SD702 lipase promoter region and signal sequence (see FIG. 10)

SD702 promoter region and signal sequence were amplified by PCR using primer 36 and 83 containing a *Nhe* I site and pUC119SDL195+SDL451 as a template to give a *Nhe* I site at N-terminal of mature wild-type *Ps. pseudoalcaligenes* lipase. An amplified fragment was digested with *Eco* RI and *Bam* HI and cloned into pUC119 (36+83pUC). pNovo Lip/Lim was digested with *Nhe* I and *Bam* HI and the smaller fragment among the obtained 2 fragments was isolated. 36+83pUC was also digested with *Nhe* I and *Bam* HI and the larger fragment was ligated to the *Eco* RI and *Bam* HI fragment of pNOVO Lip/Lim. The constructed plasmid was termed LMNBpUC.

<u>Construction of module shifted *Ps. pseudoalcaligenes* lipase variant genes in *E. coli*</u>

<u>2.a</u>. DNA fragment was amplified using primer36 and XHO-A with pKpnINdeIBEN2 as a template.

An approximate 1.3kbp of the PCR fragment was digested with *Xho* I and isolated from agarose gel. LMNBpUC was also digested with *Xho* I and ligated to the fragment. The ligation mixture was used to transform *E. coli* JM101 and the transformant harboring the plasmid was isolated. The resulting plasmid was Termed 5apUC.

<u>2.b.</u> DNA fragment was amplified using primer36 and HPA-A with LMNBpUC as a template.

The obtained fragment was digested with *Eco* RI and *Hpa* I and a 1.8kbp fragment was isolated from agarose gel. pKpnINdeIBEN2 was also digested with *Eco* RI and *Hpa* I, ligated to the fragment and introduced into *E. coli* JM101. The obtained transformant harboring the plasmid was isolated. The resulting plasmid was Termed 5abpUC

<u>2.c</u>. A DNA fragment was amplified using primer 40 and HPA-S with LMNBpUC as a template.

The fragment was digested with *Hpa* I and *Bam* HI and a 1.1kbp fragment was isolated from agarose gel. pKpnINdeIBEN2 was also digested with *Hpa* I and *Bam* HI and ligated to the fragment and transformed into *E. coli* JM101. The obtained transformant harboring the plasmid was isolated. The resulting plasmid was Termed 5cpUC.

<u>2.d.</u> pKpnINdeIBEN2 was digested with *Sac* II and *Nde* I and the smaller fragment among the resulting 2 fragments was isolated. LMNBpUC was also digested with *Sac* II and *Nde* I and the larger fragment covering vector region was ligated to it. The ligation mixture was used to transform *E. coli* JM101 and transformant harboring the expected plasmid was isolated. The resulting plasmid was Termed 5abcpUC

<u>Construction of pMLM3R</u>

pLMNBpUC was digested with *Hind* III and the smaller fragment among the obtained 2 fragments was isolated. The fragment was transferred into *Hind* III site of pMES6. The constructed plasmid was termed pMLM3R.

<u>Transfer into *Pseudomonas* expression plasmid (see FIG. 13)</u>

The constructed plasmids described above were digested with *Nhe* I and *Bam* HI and the smaller fragments among the obtained 2 fragments were isolated and ligated to the 12kbp of *Nhe* I-*Bam* HI fragment of pMLM3R The ligation mixtures were used to transform *E. coli* JM101 and each transformants harboring the plasmids were isolated.

The constructed plasmids were termed as follows: pLMV5a, pLMV5ab, pLMV5c, pLMV5abc.

EXAMPLE 7

Construction of expression plasmids of site-directed *Ps. pseudoalcaligenes* lipase mutants

Construction of site-directed *Ps. pseudoalcaligenes* lipase mutant genes in *E. coli*

1.a. A DNA fragment was amplified using primer36 and D229G+230F with LMNBpUC as a template. LMNBpUC was digested with *Sac* II and *Bam* HI and the resulting smaller fragment was isolated. The amplified fragment and *Sac* II-*Bam* HI fragment were mixed, boiled for 5 minutes and cooled slowly till room temperature for annealing. Then *Taq* polymerase was added and incubated at 72°C for 10 minutes for elongation. Using primer 43 and 27, a 0.7kbp fragment was amplified by PCR. The fragment was digested with *Kpn* I and *Nde* I and a 0.3kbp fragment was isolated from agarose gel. LMNBpUC was also digested with *Kpn* I and *Nde* I and ligated to the fragment.

The resulting plasmid was termed 229pUC.

1.b. A DNA fragment was amplified using primer36 and D250N with LMNBpUC as a template. Following the same procedure as described in 1.a. a plasmid named 250pUC was constructed.

1.c. A DNA fragment was amplified using primer 36 and D272S with LMNBpUC as a template. With the same procedure as described in 1.a., a plasmid named 272pUC was constructed.

1.d. 229pUC was digested with *Xho* I and the smaller fragment among the resulting 2 fragments was isolated. 250pUC was also digested with *Xho* I and the larger fragment corresponding to vector region was ligated to it. The resulting plasmid was termed 229.250pUC.

1.e. 250pUC was digested with *Nhe* I and *Nsp* V and the smaller fragment among the resulting 2 fragments was isolated. 272pUC was also digested with *Nhe* I and *Nsp* V and the larger fragment was ligated to the fragment. The resulting plasmid was termed 250.272pUC.

1.f. 229pUC was digested with *Xho* I and the smaller fragment among the resulting 2 fragments was isolated. 250.272pUC was also digested with *Xho* I and the larger fragment was ligated to it. The resulting plasmid was termed 229.250.272pUC.

<u>Transfer into *Pseudomonas* expression plasmid</u>

The constructed plasmids described above were digested with *Nhe* I and *Bam* HI and the smaller fragments among the resulting 2 fragments were isolated. pMLM3R was partially digested with *Nhe* I and *Bam* HI and a 12kbp NheI-BamHI vector fragment was ligated to them, and transformed into *E. coli* JM101. The obtained transformants harboring the plasmids were isolated. The constructed plasmids were termed as follows:

pLMV-D229G+230F, pLMV-D250N, pLMV-D272S, pLMV-229.250, pLMV-250.272, pLMV-229.250.272.

EXAMPLE 8

Construction of random mutagenized libraries

<u>Random mutagenized libraries of the entire *Ps. pseudoalcaligenes* lipase gene coding a lipolytic enzyme by error prone PCR method</u>

A random mutagenized library of *Ps. pseudoalcaligenes* lipase was constructed by error prone PCR. The PCR was performed under the following condition with pWYLM as a template.

10 µl	10× reaction buffer (Boehringer)
10 µl	DMSO
0.1 µl	β-mercapt ethanol
1 µl	primer 101 (100 pmol/µl)
1 µl	primer 127 (100 pmol/µl)
0.2 µl	dATP (100 mM)
0.2 µl	dGTP (100 mM)
1 µl	dCTP (100 mM)
1 µl	dGTP (100 mM)
0.55 µl	MgCl2 (1 M)
5 µl	MnCl2 (100 mM)
1 µl	Taq polymerase (1 U/ µl Boehringer)
1 µl	pWYLM (10 ng/µl) (template)

67.95 µl H2O

94° C 1 minute, 45° C 1 minute, 70° C 4 minutes , by 25 cycles

The amplified DNA fragments were isolated from agarose gel and digested with *Spe* I and *Apa* I. The resulting 0.7 kb fragment was ligated into the expression vector pWYLM, which had been digested with the same enzymes previously, and transformed into *E. coli* DH12S to make *E. coli* library. 300,000 clones in total were screened on olive oil assay plate (pH10) containing 0.5g/l of PCS and a positive mutant was

obtained. The mutated lipase gene was isolated and sequenced to find the following amino acid substitutions: E64K and L269P.

Localized random mutagenesis

Mutagenic primers (oligonucleotides) were synthesized which corresponds to the part of the DNA sequence to be mutagenized. Subsequently, the mutagenic primers were used in PCR reactions with suitable opposite primers. The resulting PCR fragments were purified, digested, cloned into *E. coli* expression vector, pWYLM, and transformed into *E. coli* DH12S. The *E. coli* transformants were spread onto LB plates containing ampicillin.

By using this method, 7 libraries containing from 20,000 to 200,000 clones were prepared.

Mutagenic oligonucleotide primers are the following:

107 (lower)

5'-GAA CTT GAT CAA (C/A)76 (C/G)66 (C/G)57 (A/C)66 788 (C/G)56 (G/T)58 (C/G)66 (T/A)76 (C/G)56 (G/T)58 (A/C)76 88(12) (A/C)66 (C/G)76 768 (C/G)66 (C/A)77 (C/A)77 (G/T)58 68(13) 767 (G/T)58 555 786 (C/G)76 (C/A)78 7(6/8)(12) GCT GCC TTT GTG CG-3'

108 (upper) 90mer

C GCG TTG ATC AAC 886 68(G/T) 576 77(G/T) 576 86(G/C) 576 5(C/A)(C/A) 86(C/G) 66(G/A) (10)57 556 76(A/T) 68(G/T) 77(C/G) 76(A/T) 68(G/C) (11)57 86(C/G) 68(G/T) 556 576 (11)55 77(T/G) 76T GCA GCG TTT AAC

109 (lower) 123mer

GTA CAC GGT CAC C77 7(T/G)6 (G/T)78 88(12) 655 65(C/A) (G/C)75 (T/G)78 65(C/A) (C/G)66 555 (G/T)78 68(13) 788 (C/G)56 88(12) 686 (C/G)57 787 788 658 (A/G)67 (G/A)85 586 586 (G/A)67 (A/T)58 (A/T)56 88(14) (C/G)66 (C/G)95 787 768 CGA GCA GCG GCC

110 (upper) 106mer

CAG CTG AAC ACT AGT (11)57 68(G/T) 67(C/G) 77(C/G) (11)55 (A/G/C)55 68(G/T) 68(C/G) (11)55 (10)57 78(C/G) (11)55 (11)55 58(T/A) 76(A/T) 76(A/T) 58(C/A) 576 77(C/G) 955 7(C/G)(C/G) 955 78(G/T) 556 68(C/G) 78C GGC CAC AGC CAC G-3'

111 (lower) 126mer

CAA GTG GCT CGA G65 (C/G)67 (G/C)66 (C/G)56 (C/A)57 (C/G)66 786 788 (C/A)77 68(12) 586 555 (G/T)78 (A/C)57 (G/C)(11)5 (C/G)(11)5 (C/A)76 (C/G)66 (C/G)57 (C/A)57 (C/A)57 586 768 (A/T)56 786 (C/A)57 (C/G)56 788 (G/T)78 (C/A)57 (C/A)77 (G/C)75 (G/C)78 (A/C)66 GGA CCA GCT GTA -3'

113 (lower) 120mer

5'-AG CGG GGA GGT ACC G75 (10)(10)5 768 (G/A)85 (G/A)85 768 (A/T)56 (C/G)66 788 (G/C)56 (C/T)88 (A/G)85 (A/T)76 (C/A)66 88(12) (C/G)66 (A/G)65 (G/C)76 (G/C)75 (C/G)78 (C/A)77 (G/T)58 (C/A)66 68(13) (C/A)77 (G/A)85 6(8/6)(14) (G/C)76 788 555 C(C/G)6 (T/A)76 AGC ACC TTC G -3'

BOTTLE 5	94% A, 2% G, 2% T, 2% C
BOTTLE 6	94% C, 2% G, 2% T, 2% A
BOTTLE 7	94% G, 2% A, 2% T, 2% C
BOTTLE 8	94% T, 2% G, 2% A, 2% C
BOTTLE 9	94% A, 3% G, 3% C
BOTTLE 10	94% C, 3% G, 3% A
BOTTLE 11	94% G, 3% A, 3% C
BOTTLE 12	94% C, 3% G, 3% T
BOTTLE 13	94% G, 3% C, 3% T
BOTTLE 14	94% T, 3% G, 3% C

Most of the PCR works were carried out under the following condition:

71.45µl	H_2O
10µl	10 X reaction buffer (w/o Mg)
0.55µl	1M $MgSO_4$
10µl	DMSO
1µl	Taq polymerase (1U/µl)
1µl X 2	Primers (100pmol/µl)
1µl X 4	dNTPs (100mM)
1µl	Template (pWYLM)

94° C 1 minute, 50°C 1 minute, 70°C 4 minutes , by 25 cycles

The examples of the combination of primers are shown in the following table.

Examples of the combination of primers

Primer	Restriction enzyme site	Length of the resultant fragment after restriction enzyme digestion (bp)	Targeted region for mutagenesis
101-107	*Spe* I-*BCl* I	270	D118-A145
108-104	*BCl* I-*Bst* EII	390	F149-A173
102-109	*Eag* I-*Bst* EII	460	S240-P273
110-111	*Spe* I-*Xho* I	550	E59-V84 + G206-C239
110-113	*Spe* I-*Kpn* I	450	E59-V84 + A174-S205
110-105	*Spe* I-*Pst* I	350	E59-V84
101-113	*Spe* I-*Kpn* I	450	A174-S205

The prepared libraries which had 20,000 to 200,000 clones per each were screened on olive oil assay plates with or without PCS as described below.

Examples of the screened libraries

Combination	Library size	Screening plate
108-104	200,000	0.5g/L detergent, pH10
	75,000	0g/L, pH10
102-109	140,000	0.5g/L detergent, pH10
	45,000	0g/L, pH10
	40,000	0.25g/L, pH8
110-111	180,000	0.5g/L detergent, pH10
	40,000	0.25g/L, pH8
101-107	20,000	0.5g/L detergent, pH10
	20,000	0g/L, pH10
	20,000	0.25g/L, pH8
110-105	20,000	0.25g/L, pH9
101-113	100,000	0.25g/L, pH9
110-113	40,000	0g/L, pH10
	40,000	0.25g/L, pH8

Several positives were detected, isolated and sequenced to find the following amino acid substitutions.

<u>pH10 with 0.5g/L PCS</u>

pWYLM-1 T156K, N169S

pWYLM-2 T156K

<u>pH10 without PCS</u>

pWYLM-3 T156N

pWYLM-4 T156K, S167A

pWYLM-5 T156K, P158R

These *E. coli* positives were tested on olive oil assay plates again as shown in the following table.

Some of the clones, however, seemed to decrease their activity and did not show halos on the same condition where they had been screened.

Appearance of halos of the obtained mutants under various conditions

Detergent condition (g/L)	pH	EGTA (mM)	WYLM	WYLM -1	WYLM -2	WYLM -3	WYLM -4	WYLM -5
0	8	0.5	+	++	(±)	++	++	++
		1.5	(±)	++	(+)	++	++	++
	9	0.5	(+)	++	-	+	+	++
		1.5	-	++	(+)	+	+	++
	10	0.5	-	+	-	(±)	-	+
		1.5	-	-	-	-	-	-
0.25	8	0.5	-	(+)	-	(+)	(+)	(+)
		1.5	-	+	-	+	+	++
	9	0.5	-	(±)	-	+	+	(+)
		1.5	-	-	-	-	-	(±)
	10	0.5	-	-	-	-	-	-
		1.5	-	-	-	-	-	-
0.5	8	0.5	-	-	-	(±)	(±)	+
		1.5	-	-	-	-	-	-
	9	0.5	-	-	-	-	-	-
		1.5	-	-	-	-	-	-

Construction of *Pseudomonas* vectors for random mutants

The plasmid containing *Ps. pseudoalcaligenes* lipase gene with substitutions of E64K and L269P, and T156K and N169S were digested with *Nhe* I and *Bam* HI. The smaller fragments including lipolytic enzyme gene were isolated, ligated to a 12k bp fragment of pMLM3R partially digested with *Nhe* I - *Bam* HI and transformed into *E. coli* JM101. The resulting transformant harboring the plasmid was isolated and termed as follows:

plasmid containing substitutions of E64K and L269P was termed pLMV9R

plasmid containing substitutions of T156K and N169S was termed pLMV10R

EXAMPLE 9

***Pseudomonas* transformation**

Pseudomonas mendocina LDM1 was transformed as described in the materials and methods section herein with the constructed expression plasmids for *Pseudomonas* described above. The expression of *Ps. pseudoalcaligenes* lipase variants in the transformants was tested by plate assay as described in the materials and methods section. All transformants expressed lipolytic enzyme activity on L plate containing olive oil and brilliant green.

EXAMPLE 10

Cultivation of *Pseudomonas* transformants in small scale

Pseudomonas transformants were cultivated in a tube as described in the materials and methods section herein. Culture broth was centrifuged and lipolytic activity in the supernatant was measured. 30LU/ml of lipolytic activity was produced in the supernatant of pLMV3R.

EXAMPLE 11

Screening for washing test

All the cultures of the *Pseudomonas* transformants harboring the constructed plasmids above were tested on various detergent concentrations and pHs to be selected for the next washing test. The following table shows some of the results.

Activity of Pseudomonas cultures of mutants under various conditions

Plasmid		pH 7						pH 8						pH 9						pH 10					
		0	0.1	0.3	0.5	0.75	1	0	0.1	0.3	0.5	0.75	1	0	0.1	0.3	0.5	0.75	1	0	0.1	0.3	0.5	0.75	1
pMES6	10LU/ml	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+	-	-	-	-	-	-	-	-	-
pMLM1	10LU/ml	(+)	(+)	(+)	-	-	-	(+)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pLMV3R	5LU/ml	(+)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pLMV5R	10LU/ml	++	++	++	++	++	++	++	++	++	++	++	++	++	(+)	-	-	-	-	(+)	-	-	-	-	-
pLMV8R	10LU/ml	++	++	++	++	++	+	++	++	++	+	(+)	(+)	+	(+)	-	-	-	-	-	-	-	-	-	-
pLMV4R	10LU/ml	++	++	++	++	(+)	(+)	++	++	++	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pLMV7R	10LU/ml	++	++	++	++	++	(+)	++	++	++	++	-	+	+	-	-	-	-	-	-	-	-	-	-	-
pHL2	10LU/ml	++	++	++	++	(+)	(+)	++	++	++	+	-	-	(+)	-	-	-	-	-	-	-	-	-	-	-
pHL4	10LU/ml	++	++	++	++	++	++	++	++	++	++	++	++	++	+	-	-	-	-	(+)	-	-	-	-	-
pLMV1R	1LU/ml	(+)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pLMV6R	10LU/ml	++	+	(+)	(+)	-	-	+	+	-	-	-	-	(+)	-	-	-	-	-	-	-	-	-	-	-

EXAMPLE 12

Jar fermentation of *Pseudomonas* transformants

Fermentation of module shifted variants and mutants

One loop of frozen cells of *Pseudomonas* mutants was inoculated on LB plate containing 20 ppm of kanamycin and grown at 30°C overnight. One loop of the cells was inoculated in a shake flask containing 100 ml of GYR medium and cultivated at 30°C overnight. The 20ml of the culture was inoculated to a 1-liter tank with C9R medium. The tank fermentation was carried out for 3 days at 30°C, keeping the agitation rate at 800rpm and pH at 8 by feeding 12.5% ammonia water and air at 0.5 air/L/min. Tween80 was added continuously to the tank at the rate of 2 g/hour.

Lipase activity of the broth of one of *Pseudomonas* transformants was about 600,000 LU/liter in 72 hours.

GYR medium:

10 g/L	Rape seed oil
20 g/L	Sodium glutamate
5 g/L	K_2HPO_4
5 g/L	Yeast extract
5 g/L	$MgSO_4 \cdot 7aq$
2.5ml/L	Trace metal compounds, cf below
2 g/L	Na2CO3
20 mg/L	Kanamycin

Trace metal compounds:

1.76 g/L	$ZnSO_4 \cdot 7aq$
0.78 g/L	$CuSO_4 \cdot 5aq$
0.82g/L	$CoSO_4 \cdot 7aq$
0.1 g/L	$Na_2MoO_4 \cdot 2aq$
0.012 g/L	H_3BO_3
2 g/L	$FeSO_4 \cdot 7aq$
2 g/L	$MnSO_4 \cdot 7aq$

C9R medium:

5 g/L	Rape seed oil
1 g/L	Tween80
2 g/L	$(NH_4)_2SO_4$
10 g/L	K_2HPO_4
4 g/L	KH_2PO_4
3 g/L	$MgSO_4 \cdot 7aq$

0.5g/L	$CaCl_2 \cdot 2aq$
0.3 g/L	$FeSO_4 \cdot 7aq$
0.05 g/L	$MnSO_4 \cdot 4\text{-}5aq$
0.05 g/L	$ZnSO_4 \cdot 7aq$
2.7 g/L	Na_2CO_3
20 mg/L	Kanamycin

EXAMPLE 13

Purification of *Pseudomonas* lipolytic enzyme variants

Pseudomonas cultures were purified as described in **method**.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: NOVO NORDISK A/S

(B) STREET: Novo Allé

(C) CITY: Bagsvaerd

(E) COUNTRY: Denmark

(F) POSTAL CODE (ZIP): DK-2880

(G) TELEPHONE: +45 4444 8888

(H) TELEFAX: +45 4449 3256

(ii) TITLE OF INVENTION: NOVEL LIPOLYTIC ENZYMES

(iii) NUMBER OF SEQUENCES: 29

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1992 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GCTAGCTTGT TCGGTTCCAC CGGTTACACC AAAACCAAGT ACCCGATCGT TCTGACCCAC 60

GGCATGCTCG GTTTCGATAG CATCTTGGGC GTGGACTACT GGTACGGCAT CCCGTCCAGC 120

TTGCGTAGCG ACGGCGCATC CGTCTACATT ACCGAGGTGT CCCAGCTGAA CACTAGTGAG 180

CTGCGCGGCG AAGAACTGCT CGAACAGGTC GAAGAAATTG CAGCAATCAG CGGCAAAGGC 240

AAAGTGAACC TCGTCGGCCA CAGCCACGGT GGCCCGACGG TGCGCTACGT CGCCGCGGTG 300
CGCCCGGACC TCGTGGCGTC CGTTACGTCC GTCGGCGCAC CGCACAAAGG CAGCGACACG 360
GCCGACTTTA TCCGCCAGAT CCCGCCGGGC AGCGCCGGTG AAGCTATCGT GGCAGGCATC 420
GTCAACGGTC TCGGCGCGTT GATCAACTTC CTGAGCGGGA GCTCCAGCAC CTCCCCGCAG 480
AACGCACTGG GCGCGCTGGA GTCCCTGAAC AGCGAAGGTG CTGCAGCGTT TAACGCGAAG 540
TACCCGCAGG GTATCCCGAC CTCCGCGTGC GGCGAAGGTG CATACAAGGT CAACGGCGTT 600
AGCTACTACA GCTGGTCCGG TACCTCCCCG CTGACCAACG TGCTGGACGT TAGCGATTTG 660
CTCCTCGGCG CGTCCTCCCT GACCTTTGAT GAGCCGAACG ACGGCCTGGT GGGCCGCTGC 720
TCGAGCCACT TGGGCAAAGT TATTCGCGAT GATTACCGTA TGAACCACCT CGACGAAGTG 780
AACCAGACCT TTGGCCTGAC CTCCCTGTTC GAAACCGACC CGGTGACCGT GTACCGTCAG 840
CAGGCAAACC GCCTGAAACT CGCCGGTCTG TGAGCCATAG ATCGGGGCCC ACAGGCCTCG 900
ATATTTTCCC CCGAGGAGTC TCCATATGAA CAAAGCGCTG CTCCTCGCCG TGCCGCTGCT 960
CATCGGCGCG GGTATCGCAG TCACCCTGGC ACTCAACCCG CTGACCCCGG CACCGTCCCC 1020
GGCGGCACTC TCGACCGCAC CGGGCGTCCC GCTGCCGAGC CCGGCGGTCC AGCGCACCCT 1080
GGACGACGCC CCGGCGGCCC CGCCGCTGGC CGCCGAAATT GCGCCGTTGC CGCCGAGCTT 1140
TGCCGGTACG CAGGTGGATG GCCAGTTTCG CCTCGATGCG GCGGGCAACC TGCTGATTGA 1200
GCGCGACATC CGCCGCATCT TCGACTACTT TCTGAGCGCA TACGGCGAGG ATTCCCTTAA 1260
GGCCACCATT GAGCGTCTCC AGGCATACGT TCGCAGCCAG TTGGACGAGC CGGCAGAGAG 1320
CCAGGCACTG GCATTGCTGG AACAGTACCT GGAGTACAAG CGTCAGCTCG TCCAGCTCGA 1380
AAAGGACCTG CCGCAGATGG CGAGCTTGGA TGCCCTCCGC CAGCGCGAAC AGGCGGTGCA 1440
GAACCTGCGC GCGTCCCTCT TCTCCGTGGA GGCGCACCAG GCGTTCTTTG CAGAGGAGGA 1500
GGCGTACAAC GGTTTTACGC TCCAGCGTCT GGCAATCCGC CATGACCAGA CGCTGGACGA 1560
CCAGCAGAAA GCAGAAGCAC TCGACCGCCT GCGTGCCAGC CTGCCGGAGG AGCTGCAAGC 1620
ATTGCTGGCA CCGCAGCTCC AGGCGGAACT GCGCCAGCAG ACCGCCGCCC TCCAGGCGCA 1680
GGGCGCGTCC GCTGCACAGA TCCAGCAGCT GCGCCTCCAG CTCGTGGGTG CCGAAGCGAC 1740
CGCGCGCCTG GAAGCGCTGG ATCAGCAGCG CCAGCAGTGG CGTCAGCGCC TGGCCGACTA 1800
CCGCCGCGAG AAAGCTCGCG TGCTGGCCAA CGATGGCCTG AGCGAAAGCG ACAAACAGGC 1860
TGCAATCGCC GAGCTGGCAG CCCAGCGCTT CGACGACAAC GAACGCCTGC GCTTGGAAGC 1920
GGCAGAACAG CTGGCGCAGT CCCGTGAGGA AAAACCGTGA GAATTCAAGC TTTCCGGAGG 1980
AGGAGGATCC GC 1992

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 946 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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GCTAGCTTCG GCTCCTCCAA CTACACCAAG ACCCAGTACC CGATCGTGCT GACCCACGGT    60
GTTTTGGGTT TTGACTCCCT GCTGGGTGTC GACTACTGGT ACGGTATCCC GTCCGCGCTG   120
CGCAAAGATG GTGCCACGGT TTACGTGACC GAAGTGAGCC AGCTCGACAC TAGTGAAGCA   180
CGCGGTGAAC AGCTGCTGAC CCAGGTGGAG GAAATCGTGG CCATCTCCGG CAAACCGAAA   240
GTGAACTTGT TCGGTCACAG CCACGGCGGC CCGACCATCC GCTACGTGGC CGCGGTTCGC   300
CCGGACCTGG TCGCCTCCGT CACGAGCATC GGGGCCCCGC ACAAGGGTTC CGCGACGGCG   360
GATTTCATCC GCCAGGTGCC GGAAGGTAGC GCATCCGAGG CGATTCTGGC CGGCATCGTG   420
AACGGTTTGG GCGCACTCAT CAACTTTCTG AGCGGGAGCT CCAGCGACAC CCCGCAGAAC   480
TCCCTGGGCA CGCTGGAGAG CCTGAATTCC GAGGGCGCAG CGCGCTTCAA CGCGCGCTTC   540
CCGCAGGGCG TCCCGACGAG CGCATGCGGC GAGGGCGACT ACGTGGTGAA CGGTGTGCGC   600
TACTACAGCT GGAGCGGTAC CTCCCCGCTG ACCAACGTGC TCGATCCGAG CGACCTCCTG   660
CTGGGCGCAA CCTCCCTGAC CTTCGGCTTT GAAGCAAACG ATGGCCTGGT CGGTCGCTGC   720
AGCTCCCGCC TCGGTATGGT GATCCGCGAT AACTACCGCA TGAACCACCT CGACGAGGTT   780
AACCAGACCT TCGGCTTGAC CAGCATTTTT GAGACCAGCC CGGTTTCCGT CTACCGCCAG   840
CAGGCCAACC GCCTGAAGAA CGCAGGTCTG TGATATAGGC TCCACAACCA GACAGGCCTG   900
GCCCCTCAGG GGCCAGTCAC AGGAGATCGA TATCCATATG GGATCC                  946
```

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
AGCAGCGGCA CGGCGAGGAG                                                20
```

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GTTTTCCCAG TCACGAC 17

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CAGGAAACAG CTATGAC 17

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CAGCGGCAAA GGCAAAGTGA 20

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CGCCTCGAGC AGCGACCGAC CAGGC 25

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CTGGTTAACT TCGTCGAGGT GGTTCATAC 29

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GAGGTTAACC AGACCTTTGG CCTGACC 27

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CGTCGTTCGG TTCGAAACCA AAGGTCAG 28

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TCATACGGTA GTTATCGCGG ATAACTTTGC C 31

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CGGTCACCGG ACTAGTTTCG AACA 24

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

AGTACCCGAT CGTTCTGACC 20

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GCGCACCGCA CAAAGGCAGC 20

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CCCGATCTAT GGCTCACA 18

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

ACGCCGTTGA CCTTGTATG 19

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

TGCCTTTGTG CGGTGCGCCG 20

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

AGCAGCGGCA CGGCGAGGAG CAG 23

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GGGAGCTCGC TCCTACACGA A 21

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

GGGGTACCGG GCGTTCTCGA TGGTTTTT 28

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

GGGGTACCCG TCACGCGCTG TCGCGCGC 28

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GGGCATGCGC GAGCAGGCTG 20

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GGCTCCTCGA ACTACACCAA GACCCAGTAC CCGATCGTCC TGACCCACGC C 51

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

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(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

GCCTGCTCGC TAGCGGCCAG A 21

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

TGTCCTGCGG GTCCAGCGAC G 21

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

GGGGATCCCC ACTCCAGAAT CGGTG 25

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

AAAAGCTTGA TCTACGCCCC AGGACC 26

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

AAGAATTCCC ACTCCAGAAT CGGTG 25

CLAIMS

1. A variant of a parent lipase, which comprises

a) substitution of an amino acid residue at a position corresponding to one of the following positions in the mature, wild-type *Ps. pseudoalcaligenes* lipase: G1, L2, F3, G4, S5, T6, G7, K12, I15, T18, M21, L22, D25, S26, I27, L28, D31, W33, Y34, G35, S38, S39, R41, S42, D43, S46, Y48, I49, T50, E51, S53, Q54, L55, N56, T57, E59, L60, E63, E64, L66, E67, V69, E70, E71, I72, A73, I75, S76, K78, G79, V84, H88, G90, V93, Y95, V96, A98, V99, D102, V110, G111, A112, P113, H114, D118, T119, D121, F122, I123, Q125, I126, P128, A131, G132, E133, A134, I135, V136, A137, G138, V140, G142, L146, F149, S153, S155, T156, S157, A161, L162, G163, A164, E166, S167, N169, S170, E171, A173, A174, A175, F176, A178, K179, Y180, Q182, I184, P185, A188, G190, E191, G192, A193, Y194, K195, V196, N197, G198, V199, S200, S205, T207, S208, P209, L210, D215, V216, S217, D218, L219, L220, G222, A223, S224, S225, T227, D229, E230, P231, D233, R238, S241, H242, K245, R248, D249, D250, R252, L256, D257, E258, V259, Q261, T262, L265, T266, S267, L268, F269, E270, D272, T275, V276, Q279, Q280, L286, A287, and L289; and/or

b) insertion of an amino acid residue between the amino acid residues located in a position of the parent lipase corresponding to positions G29 and V30; to positions I126 and P127; to positions A188 and C189; to positions A193 and Y194; to positions D229 and E230; and/or positions E230 and P231 of the wild type *Ps. pseudoalcaligenes* lipase; and/or

c) deletion of an amino acid residue at a position corresponding to one of the following positions in the mature, wild-type *Ps. pseudoalcaligenes* lipase: G1, L2, F3, G4, S5, T6, G7, I75, S155, T156, S157, L265, T266, S267, L268, F269, E270.

2. The lipase variant according to claim 1, which comprises substitution with an amino acid residue which is hydrophobic and/or positively charged at a position corresponding to one of the following positions in the mature, wild-type *Ps. pseudoalcaligenes* lipase: I15, M21, I27, L28, D31, W33, Y34, S38, S39, S42, S46, Y48, I49, T50, S53, Q54, L55, L60, L66, E67, V69, I72, A73, S76, G79, V84, V93, Y95, V96, V99, V110, A112, P113, D118, T119, F122, I123, Q125, I126, A131, G132, A134, I135, V136, G138, V140, G142, L146, A161, L162, F176, Y180, Q182, I184, G190, A193, Y194, K195, V196, N197, V199, S200, S208, P209, L210, V216, S217, L219, L220,

G222, A223, S225, T227, P231, H242, V259, Q261, T262, T266, S267, L268, V276, Q280, A287 or L289.

3. The lipase variant according to claim 1, which comprises substitution with a neutral or positively charged amino acid residue at a position corresponding to position D25, D31, D43, E51, E59, E63, E64, E67, E70, E71, D102, D118, D121, E166, E171, E191, D215, D229, E230, D233, D249, D250, D257, E258 and/or D272 in the mature, wild-type *Ps. pseudoalcaligenes* lipase.

4. The lipase variant according to claim 1, which comprises at least one, and preferably more of the following substitutions: G1A, G1S, L2W, T6S, G7N, K12Q, I15V, T18V, M21L, M21T, M21V, M21I, L22T, D25N, S26T, I27L, L28G, D31R, D31N, W33F, Y34H, G35T, S38W, S39A, S39N, R41E, S42K, S42R, S46R, S46T, Y48H, I49V, T50A, E51S, S53A, Q54A, L55F, N56D, T57D, L60A, L60Q, E63A, E64K, E64Q, L66A, E67R, E67T, V69I, E70V, E71P, I72W, A73V, S76G, K78G, G79P, V84F, V84I, H88Q, G90S, V93I, V93S, Y95V, V96A, A98S, V99L, V110I, G111N, A112G, P113V, H114N, D118A, D118K, T119V, F122V, I123V, Q125G, I126V, P128E, A131G, G132S, G132I, A134G, I135G, V136A, V136L, A137N, G138A, V140A, G142A, L146V, F149L, S153G, S155N, T156D, T156K, S157T, S157N, S157I, A161S, A161G, L162I, G163N, A164T, E166G, S167T, N169S, N169T, S170T, E171A, A173T, A174S, A175R, F176L, A178S, K179R, Y180F, Y180H, Q182W, I184V, P185N, A188S, G190A, E191K, G192S, A193D, A193T, Y194V, K195R, K195V, V196G, N197H, G198S, V199I, S200R, S205T, T207N, S208A, P209A, L210Y, V216A, V216F, V216P, S217A, L219P, L220F, G222A, A223F, S224T, S225G, T227V, D229G, D229R, D229I, E230F, P231A, P231K, R238V, S241T, H242R, H242Y, K245M, K245Q, R248D, D250R, D250N, D250S, R252N, L256V, E258A, V259I, Q261H, T262L, L265I, T266R, S267G, L268I, L268P, L268W, D272E, D272S, T275S, V276L, Q280H, L286I, L286N, A287K, and L289V.

5. The variant according to claim 4 which comprises two or more variations selected among the following substitutions or insertion:

a) substitutions V216P, S224T, D229G, P231A, H242R, K245M, D250N, L268I, D272S, T275S, L286I, D272S, T275S, L286N and

b) insertion of F between D229 and E230.

6. The variant according to claim 1, which comprises at least the following substitutions:

a) M21L,T,V,I+V216F+D31R,N+E230F;

b) M21L,T,V,I+ S153G+S42K+P128E;

c) M21L,T,V,I+A175R+D250R,N;

d) M21L,T,V,I+D229R,I+V216F+D31R,N+E230F+P128E;

e) M21L,T,V,I+S157N,I+E230F+V216F;

f) M21L,T,V,I+D31R,N+S157N,I+E230F+V216F;

g) V216F+D31R,N+E230F;

h) S153G+S42K+P128E;

i) A175R+D250R,N;

j) D229R,I+V216F+D31R,N+E230F+P128E;

k) S157N,I+E230F+V216F; or

l) D31R,N+S157N,I+E230F+V216F.

7. The lipase variant according to claim 1, which comprises insertion of an L between positions corresponding to I126 and P127; an Y between positions corresponding to A188 and C189; the peptide sequence EVHN between positions corresponding to A193 and Y194; an S between positions corresponding to D229 and E230; and/or a F or S between positions corresponding to E230 and P231 of the *Ps. pseudoalcaligenes* mature, wild-type lipase.

8. The lipase variant according to any of the preceding claims, wherein the parent lipase belongs to the *Pseudomonas* sp. lipase family.

9. The lipase variant according to claim 14, wherein the parent lipase is the *Ps. pseudoalcaligenes* lipase.

10. A DNA construct encoding a lipase variant according to any of claims 1-8.

11. An expression vector harboring the DNA construct according to claim 9.

12. A host cell containing the DNA construct according to claim 9 or the expression vector according to claim 10.

13. The host cell according to claim 11, which is a cell of *E. coli* or a cell of a *Pseudomonas* sp., in particular a lipase negative strain of a *Pseudomonas* sp.

14. A method of constructing a variant lipase from a parent lipase belonging to the *Pseudomonas* sp. lipase family, which variant has an improved wash performance as compared to the parent lipase, which method comprises:

a) subjecting an amino acid subsequence region of the parent lipase which corresponds to one of the following amino acid subsequences F3-L28, 6-14, 15-18, 21-36, V30-N56, 37-39, 46-74, E57-N82, E59-V84, K80-P101, 88-100, A105-P127, 110-136, D118-A145, R124-S151, 131-152, F149-A173, 154-167, S155-Y180, 168-180, A174-S205, P181-S205, 188-200, G206-C239, S208-L235, 222-231, S240-T262, S240-P273, 258-266, L265-L289 or 267-268 of the mature, wild-type *Ps. pseudoalcaligenes* lipase to localized random mutagenesis,

b) expressing the variety of mutated DNA sequences originating from the parent lipase obtained in step (a) in suitable host cells; and

c) screening for host cells expressing a mutated lipase which has a decreased dependence on calcium and/or an improved tolerance towards a detergent or a detergent component as compared to the parent lipase.

15. The method according to claim 13, wherein the host cell is a cell of *E. coli*.

16. The method according to claim 13 or 14, wherein the screening of step (c) is performed for improved tolerance towards an anionic surfactant such as an alkyl sulfate or LAS.

17. The method according to any of claims 13-15, which further comprises expressing the DNA sequence encoding the mutated lipolytic enzyme resulting from the screening step c) in a suitable host cell and recovering the resulting mutated lipase.

18. The method according to any of claims 13-16, wherein the parent lipase is the *Ps. pseudoalcaligenes* lipase.

19. A method of producing a lipase variant according to any of claims 1-8, which method comprises culturing a host cell according to any of claims 18-19 under conditions conducive for the production of the lipase variant and recovering the lipase variant from the resulting broth.

20. A method of preparing a variant lipase, comprising:

a) selecting a first and a second *Pseudomonas* lipase having amino acid sequences with a homology above 40 %,

b) identifying substitutions, insertions and deletions whereby the second sequence differ from the first sequence,

c) designing a variant amino acid sequence by introducing one or more of said substitutions, insertions and deletions into the first amino acid sequence,

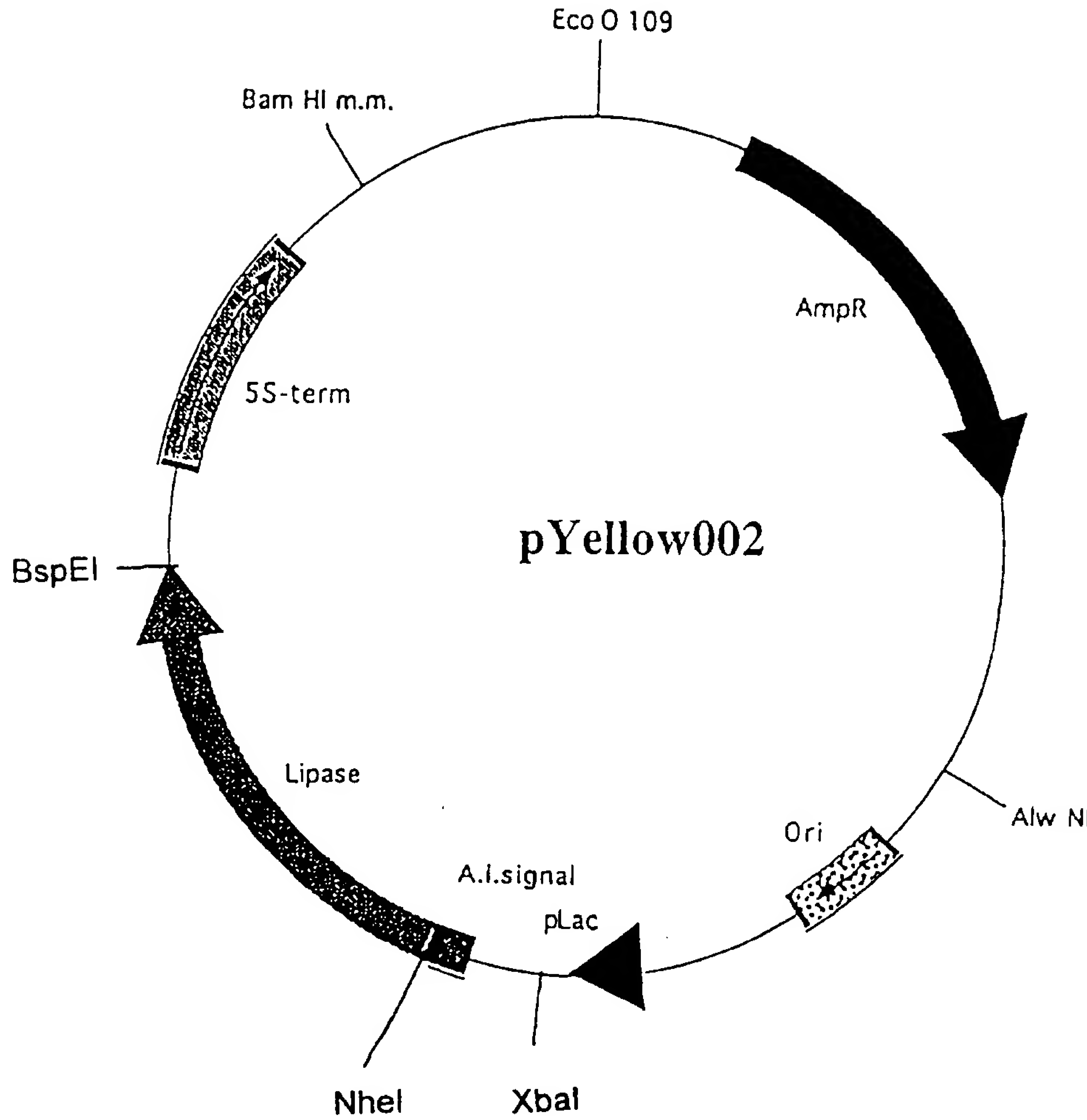
d) designing a DNA sequence expressing said variant amino acid sequence,

e) preparing said DNA sequence and transforming a suitable host organism by inserting the DNA sequence,

f) cultivating the transformed host organism to express and secrete a lipase having the variant amino acid sequence and recovering the lipase.

21. The method of claim 20 wherein the two lipases have sequences with a homology above 60 %, preferably above 80 %.

22. A detergent composition comprising a lipase variant according to any of claims 1-8 or produced according to claim 13 or 20.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Lipase
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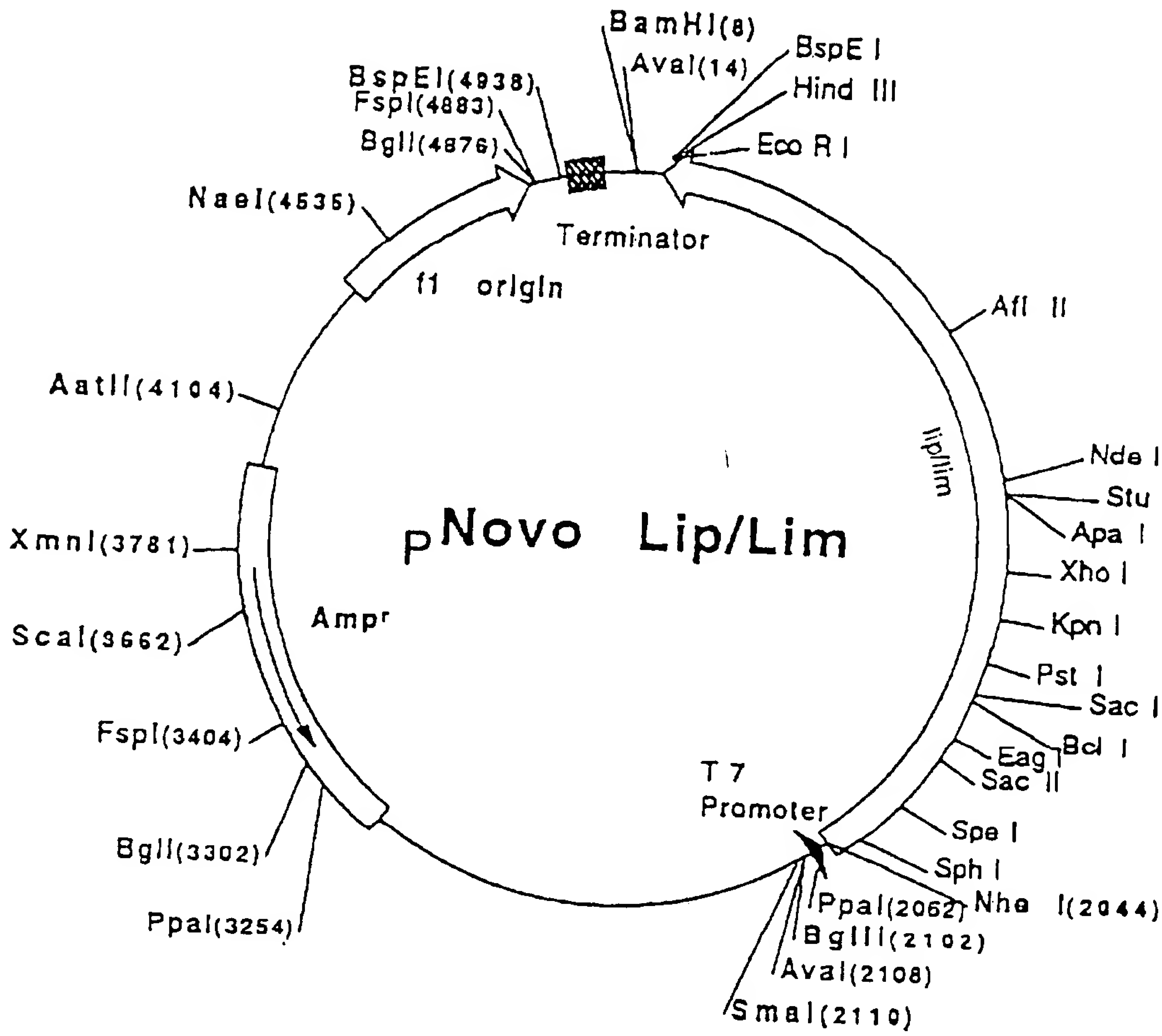
NheI
XbaI

Fig. 1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AvaI(14)
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NaeI(4535)
Terminator
f1 origin
Afl II
AatII(4104)
lip/lim
Nde I
Stu I
Apa I
pNovo Lip/Lim
XmnI(3781)
Xho I
Kpn I
ScaI(3662)
Amp^r
Pst I
Sac I
FspI(3404)
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Eag I
Sac II
T 7
Promoter
BglI(3302)
Spe I
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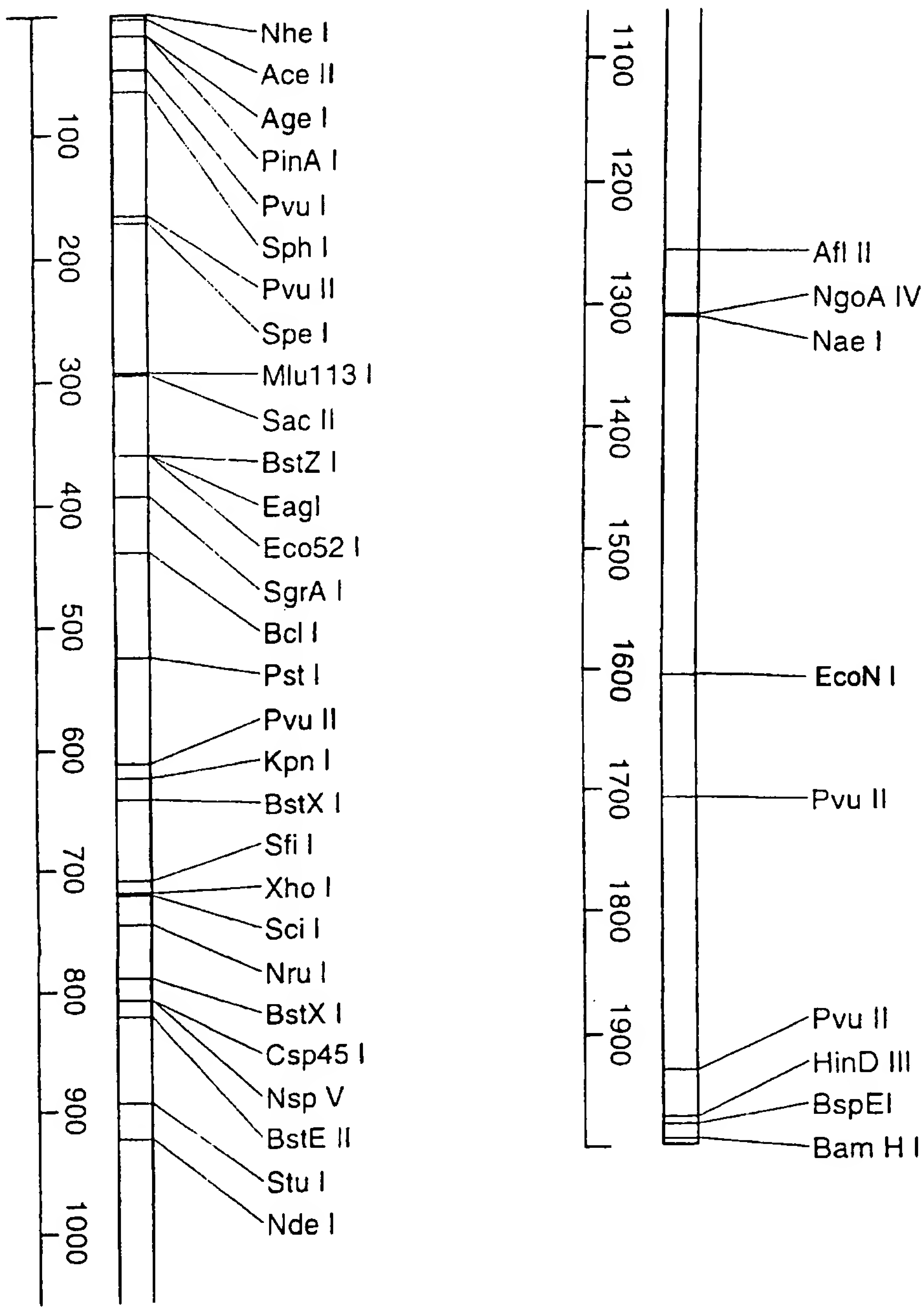
SmaI(2110)

Fig. 2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Spe I
Mlu113 I
Sac II
BstZ I
EagI
Eco52 I
SgrA I
Bcl I
Pst I
Pvu II
Kpn I
BstX I
Sfi I
Xho I
Sci I
Nru I
BstX I
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Nsp V
BstE II
Stu I
Nde I
1100
1200
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1500
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1700
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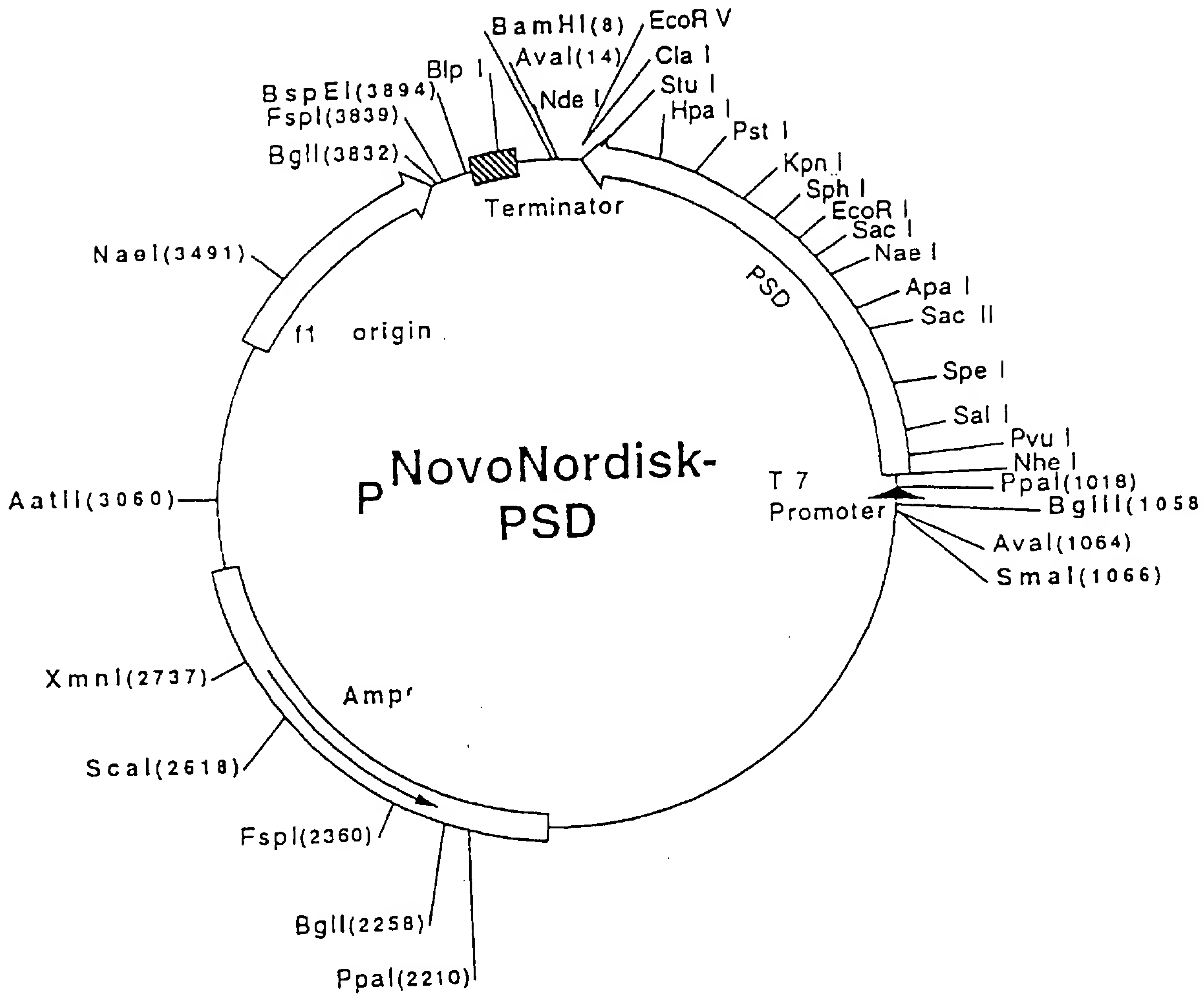
Fig. 3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Sac II
Spe I
Sal I
Pvu I
Nhe I
PpaI(1018)
BglII(1058)
AvaI(1064)
SmaI(1066)
T 7
Promoter
PSD
Terminator
Blp I
BspEI(3894)
FspI(3839)
BglI(3832)
NaeI(3491)
f1 origin
AatII(3060)
PNovoNordisk-
PSD
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Ampʳ
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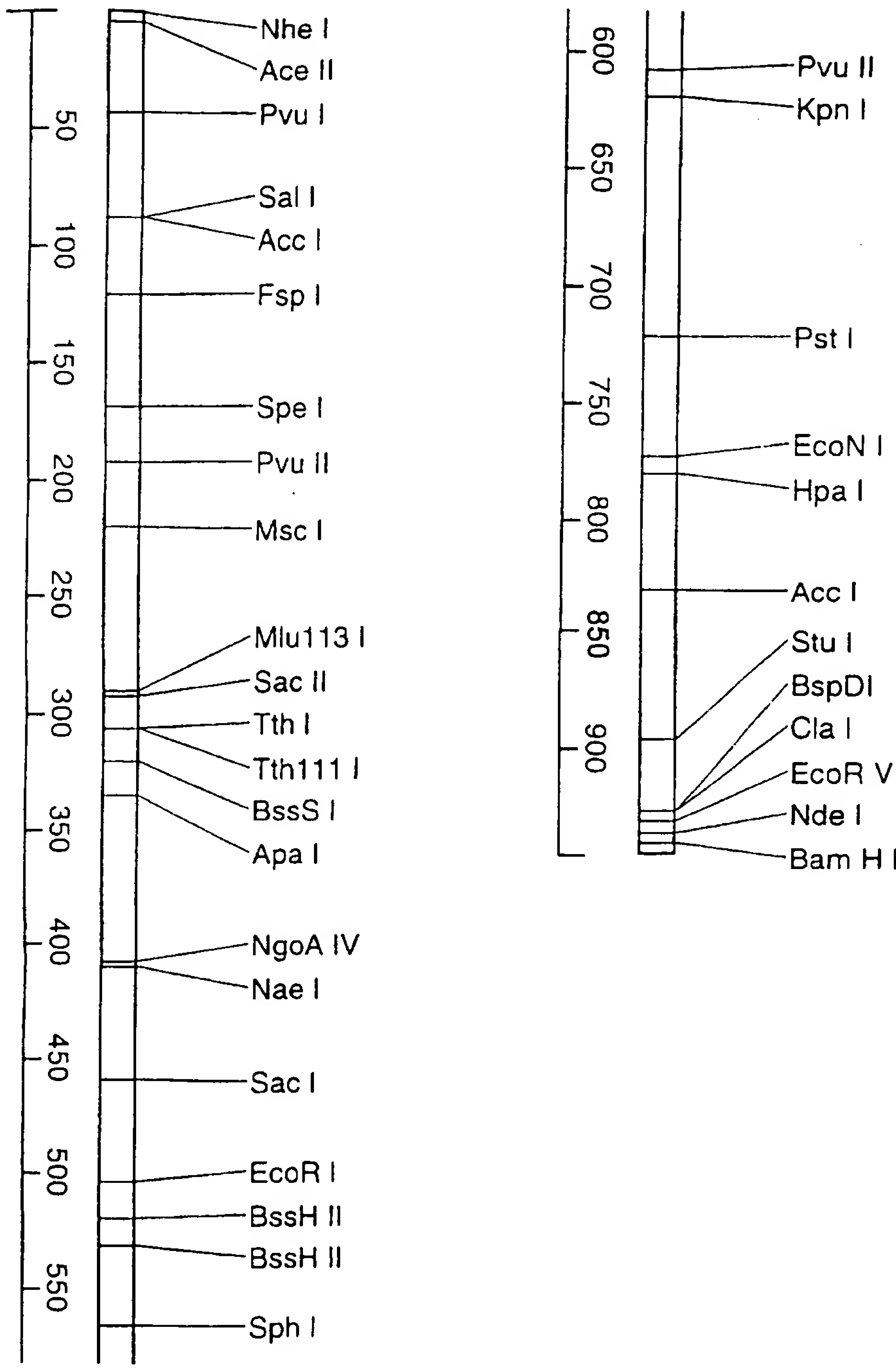
PpaI(2210)

Fig. 4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Ace II
Pvu I
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Fsp I
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Tth111 I
BssS I
Apa I
NgoA IV
Nae I
Sac I
EcoR I
BssH II
BssH II
Sph I
50
100
150
200
250
300
350
400
450
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750
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850
900
Pvu II
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Cla I
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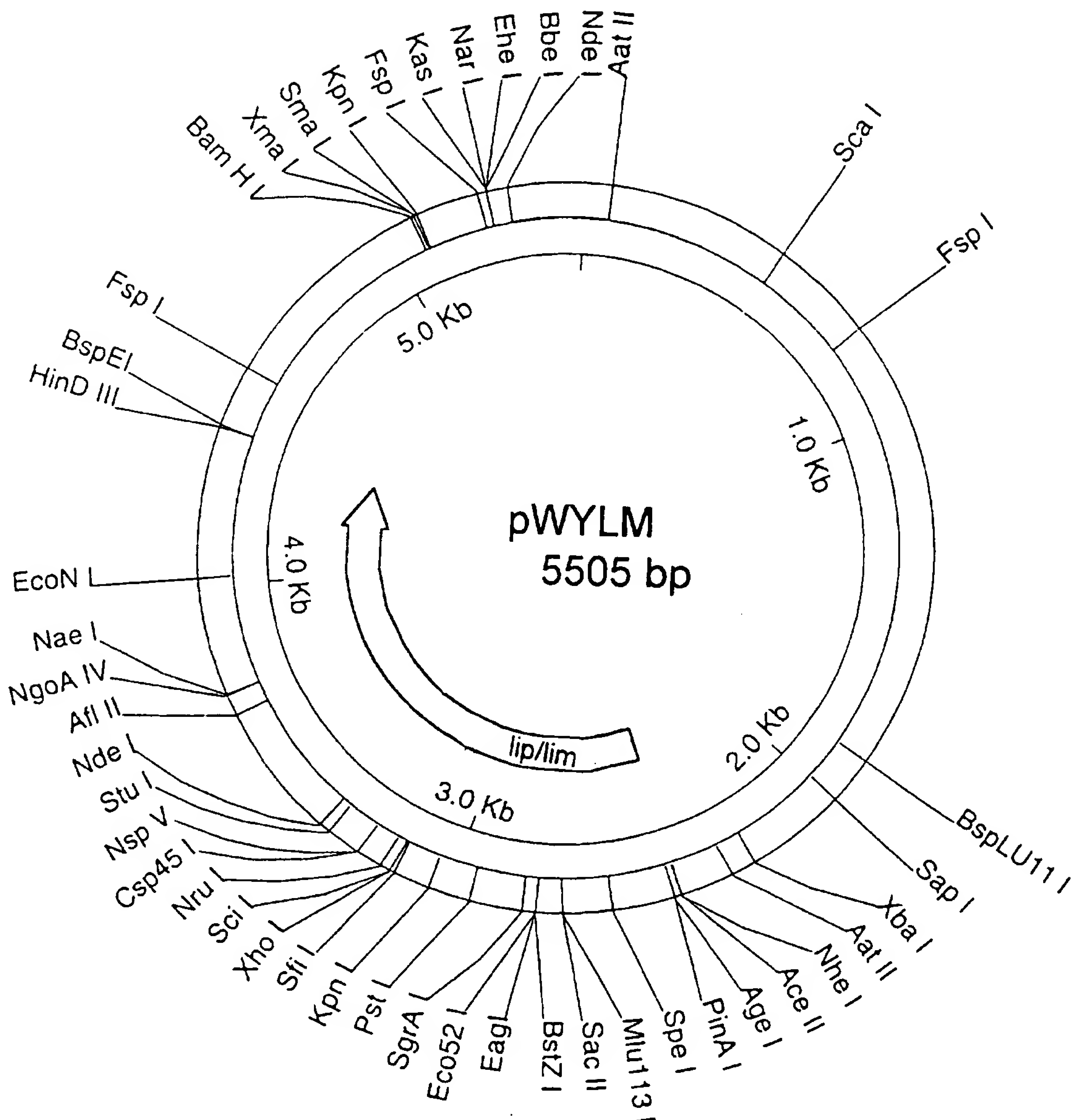
Nde I
Bam H I

Fig. 5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lip/lim
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3.0 Kb
4.0 Kb
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Aat II
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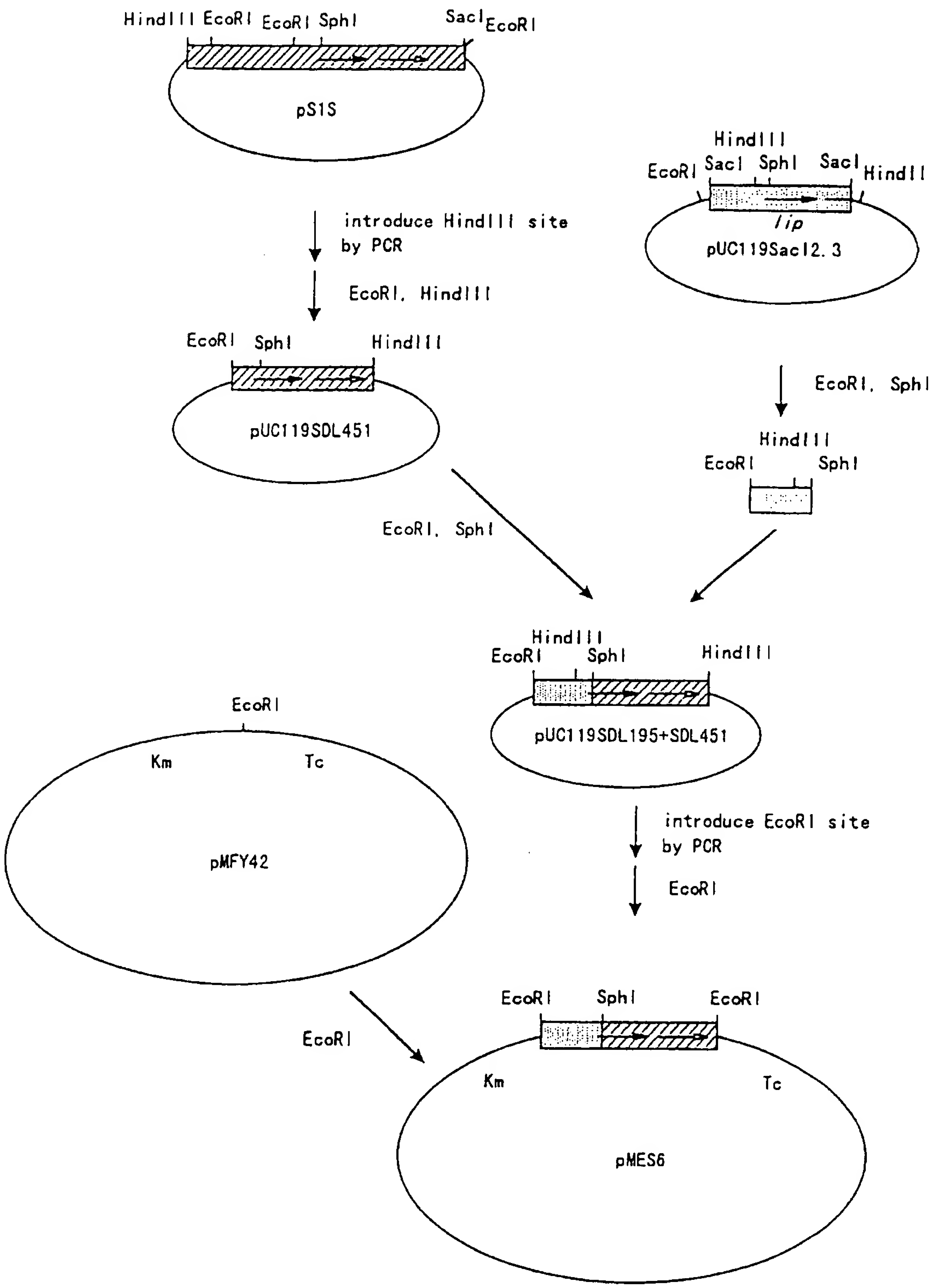
Bbe I
Ehe I
Nar I
Kas I
Fsp I
Kpn I
Sma I
Xma I
Bam H I
Sca I
Fsp I
Fsp I
BspEI
HinD III
EcoN I
Nae I
NgoA IV
Afl II
Nde I
Stu I
Nsp V
Csp45 I
Nru I
Sci I
Xho I
Sfi I
Kpn I
Pst I
SgrA I
Eco52 I
EagI
BstZ I
Sac II
Mlu113 I
Spe I
PinA I
Age I
Ace II
Nhe I
Aat II
Xba I
Sap I
BspLU11 I

Fig. 6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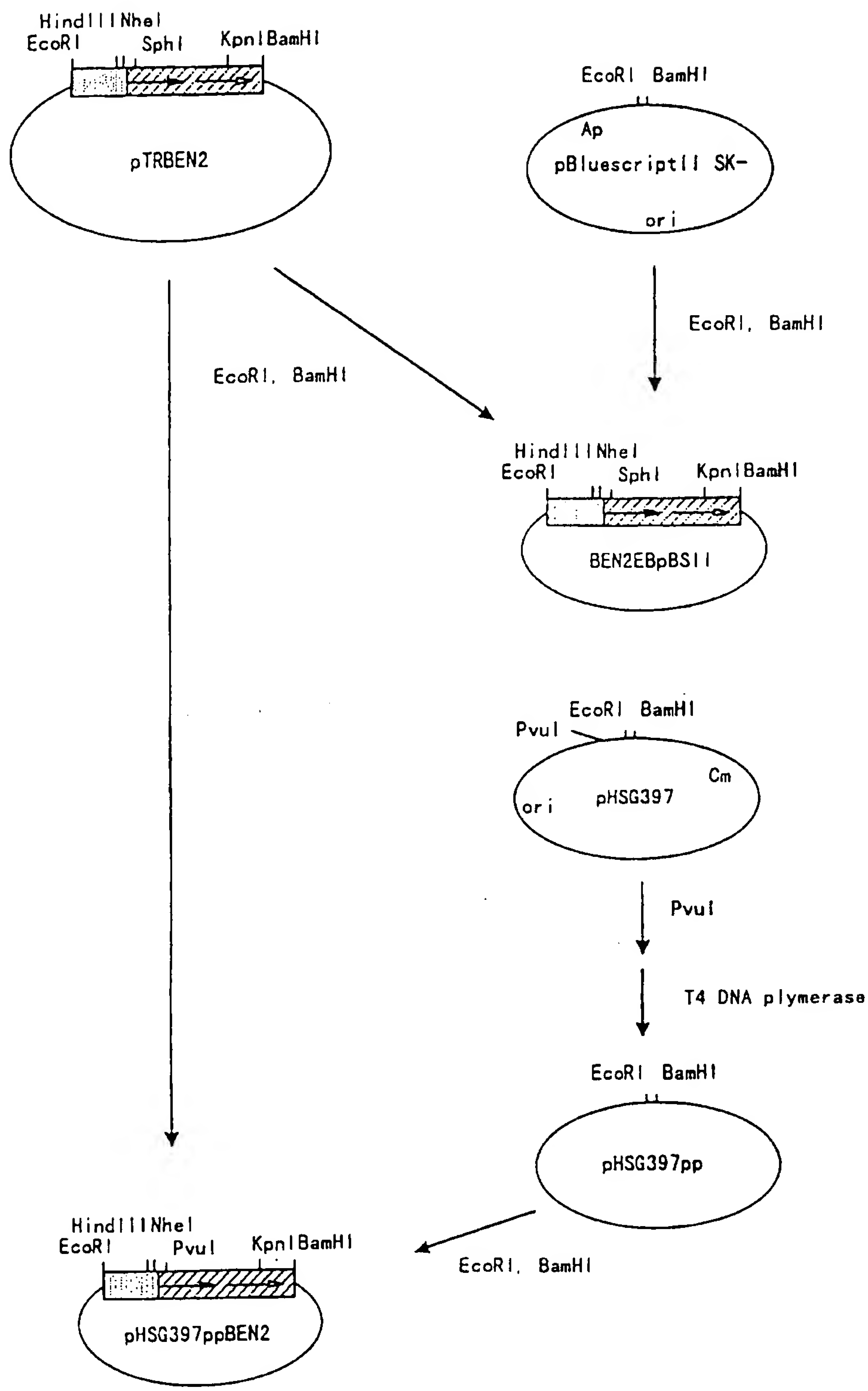
pS1S
introduce HindIII site by PCR
EcoRI, HindIII
EcoRI SphI HindIII
pUC119SDL451
HindIII
EcoRI SacI SphI SacI HindIII
lip
pUC119SacI2.3
EcoRI, SphI
HindIII
EcoRI SphI
EcoRI, SphI
HindIII
EcoRI SphI HindIII
pUC119SDL195+SDL451
EcoRI
Km Tc
pMFY42
introduce EcoRI site by PCR
EcoRI
EcoRI SphI EcoRI
EcoRI
Km Tc
pMES6

Fig. 7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EcoRI
SphI
KpnIBamHI
pTRBEN2
EcoRI BamHI
Ap
pBluescriptII SK-
ori
EcoRI, BamHI
EcoRI, BamHI
HindIIINheI
EcoRI
SphI
KpnIBamHI
BEN2EBpBSII
EcoRI BamHI
PvuI
Cm
ori
pHSG397
PvuI
T4 DNA plymerase
EcoRI BamHI
pHSG397pp
HindIIINheI
EcoRI
PvuI
KpnIBamHI
pHSG397ppBEN2
EcoRI, BamHI

Fig. 8

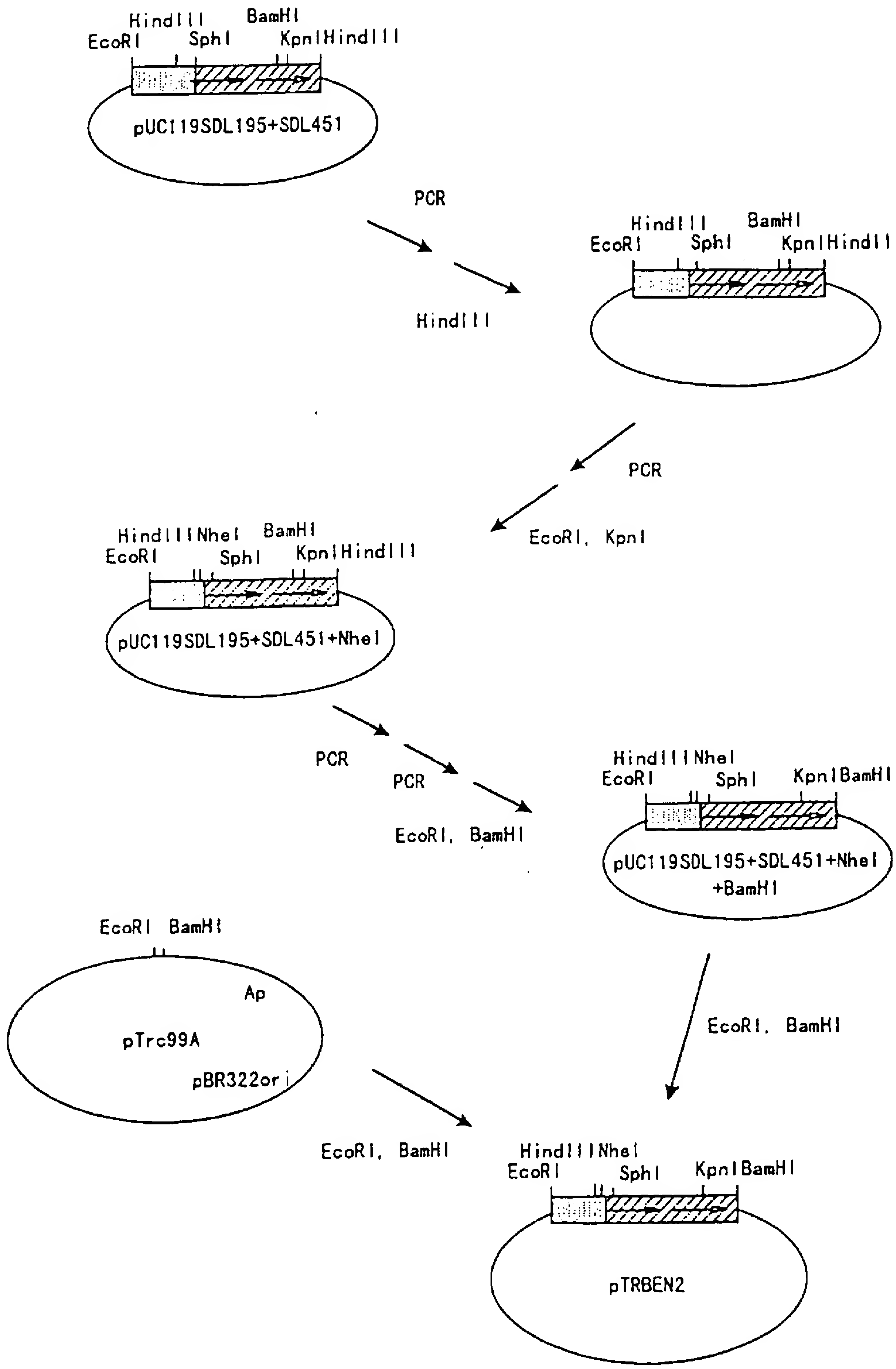

HindIII
BamHI
EcoRI
SphI
KpnI HindIII
pUC119SDL195+SDL451
PCR
HindIII
PCR
EcoRI, KpnI
HindIII NheI
pUC119SDL195+SDL451+NheI
PCR
PCR
EcoRI, BamHI
pUC119SDL195+SDL451+NheI +BamHI
EcoRI, BamHI
Ap
pTrc99A
pBR322ori
pTRBEN2

Fig. 9

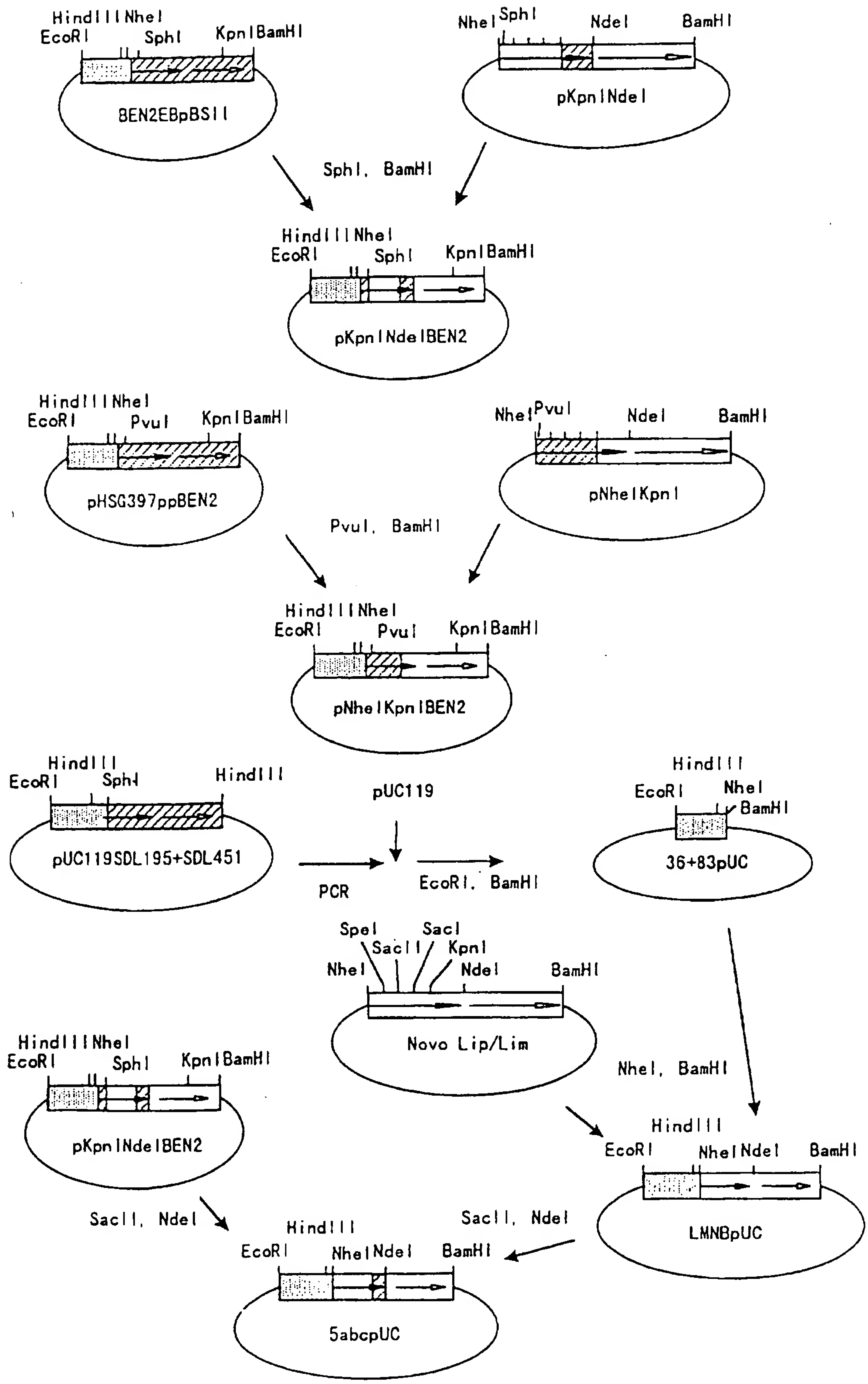
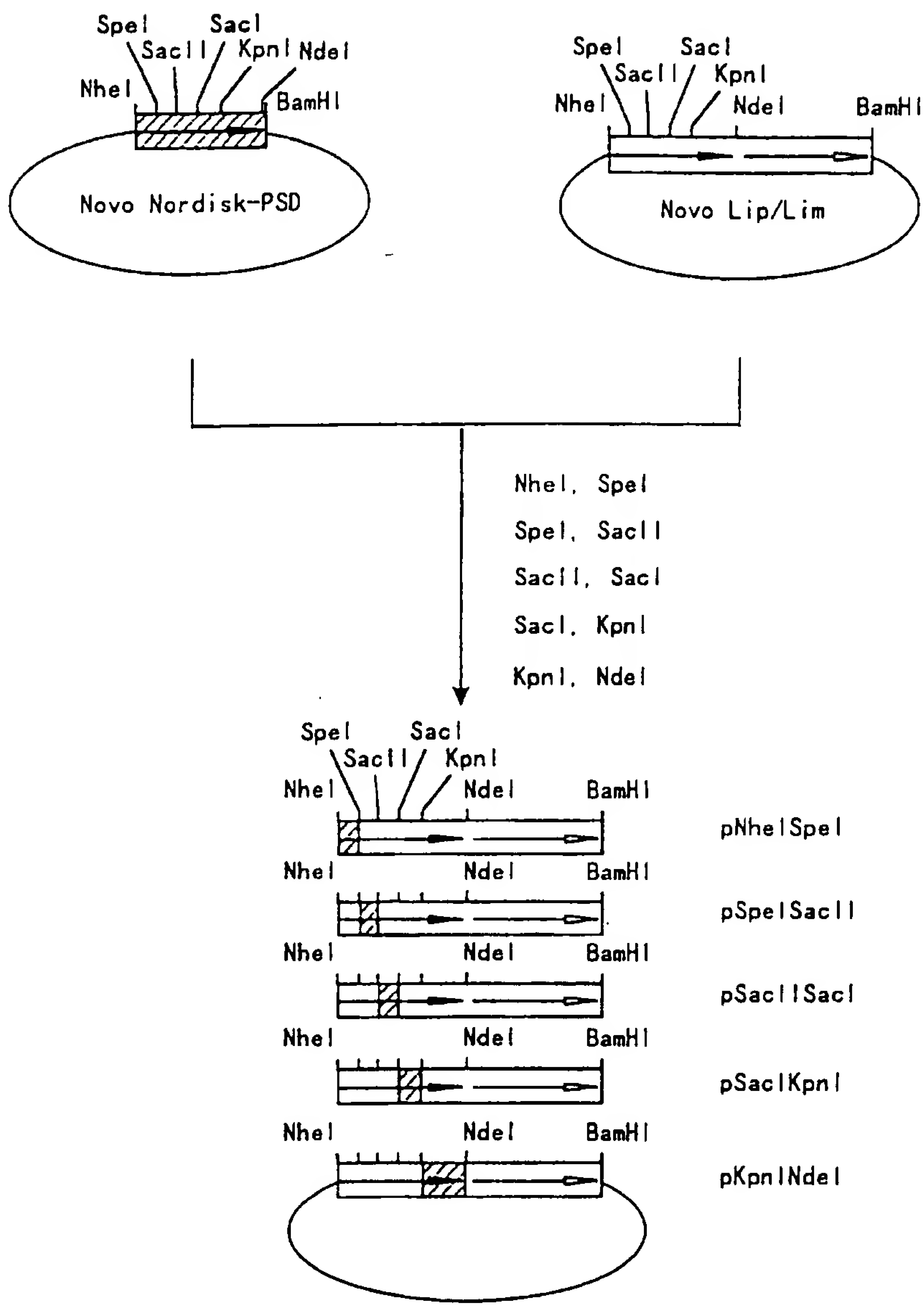

HindIIINheI
EcoRI
SphI
KpnIBamHI
BEN2EBpBSII
NheI SphI
NdeI
BamHI
pKpnINdeI
SphI, BamHI
pKpnINdeIBEN2
HindIIINheI
EcoRI
PvuI
KpnIBamHI
pHSG397ppBEN2
NheI PvuI
NdeI
BamHI
pNheIKpnI
PvuI, BamHI
pNheIKpnIBEN2
pUC119SDL195+SDL451
pUC119
PCR
EcoRI, BamHI
36+83pUC
SpeI
SacII
SacI
KpnI
Novo Lip/Lim
NheI, BamHI
LMNBpUC
SacII, NdeI
5abcpUC

Fig. 10



SpeI
SacI
SacII
KpnI
NdeI
NheI
BamHI
Novo Nordisk-PSD
SpeI
SacI
SacII
KpnI
NheI
NdeI
BamHI
Novo Lip/Lim
NheI, SpeI
SpeI, SacII
SacII, SacI
SacI, KpnI
KpnI, NdeI
SpeI
SacI
SacII
KpnI
NheI
NdeI
BamHI
pNheISpeI
NheI
NdeI
BamHI
pSpeISacII
NheI
NdeI
BamHI
pSacIISacI
NheI
NdeI
BamHI
pSacIKpnI
NheI
NdeI
BamHI
pKpnINdeI

Fig. 11

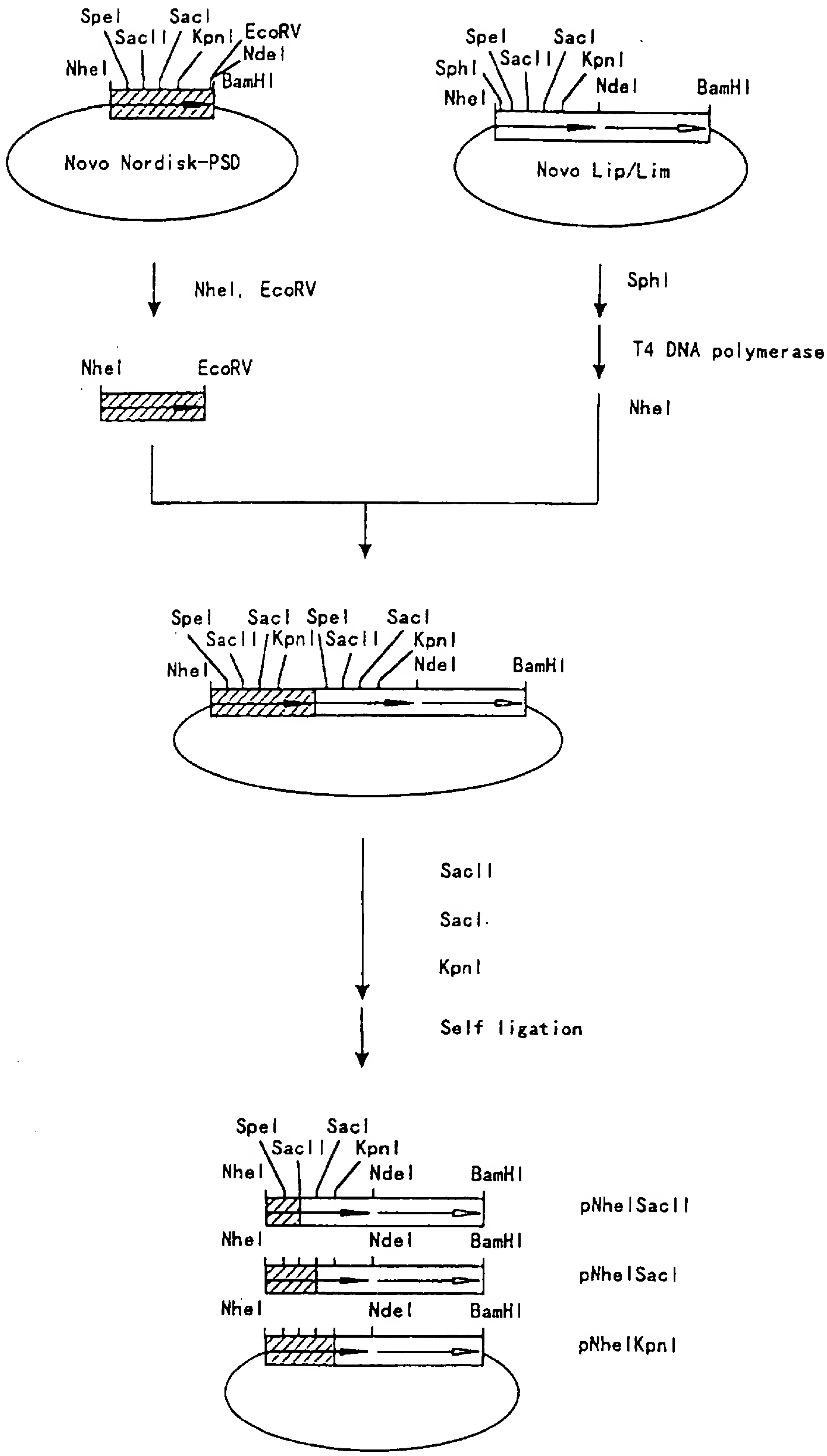

SpeI
SacII
SacI
KpnI
EcoRV
NdeI
NheI
BamHI
Novo Nordisk-PSD
SphI
Novo Lip/Lim
NheI, EcoRV
SphI
T4 DNA polymerase
NheI
EcoRV
SacII
SacI
KpnI
Self ligation
pNheISacII
pNheISacI
pNheIKpnI

Fig. 12

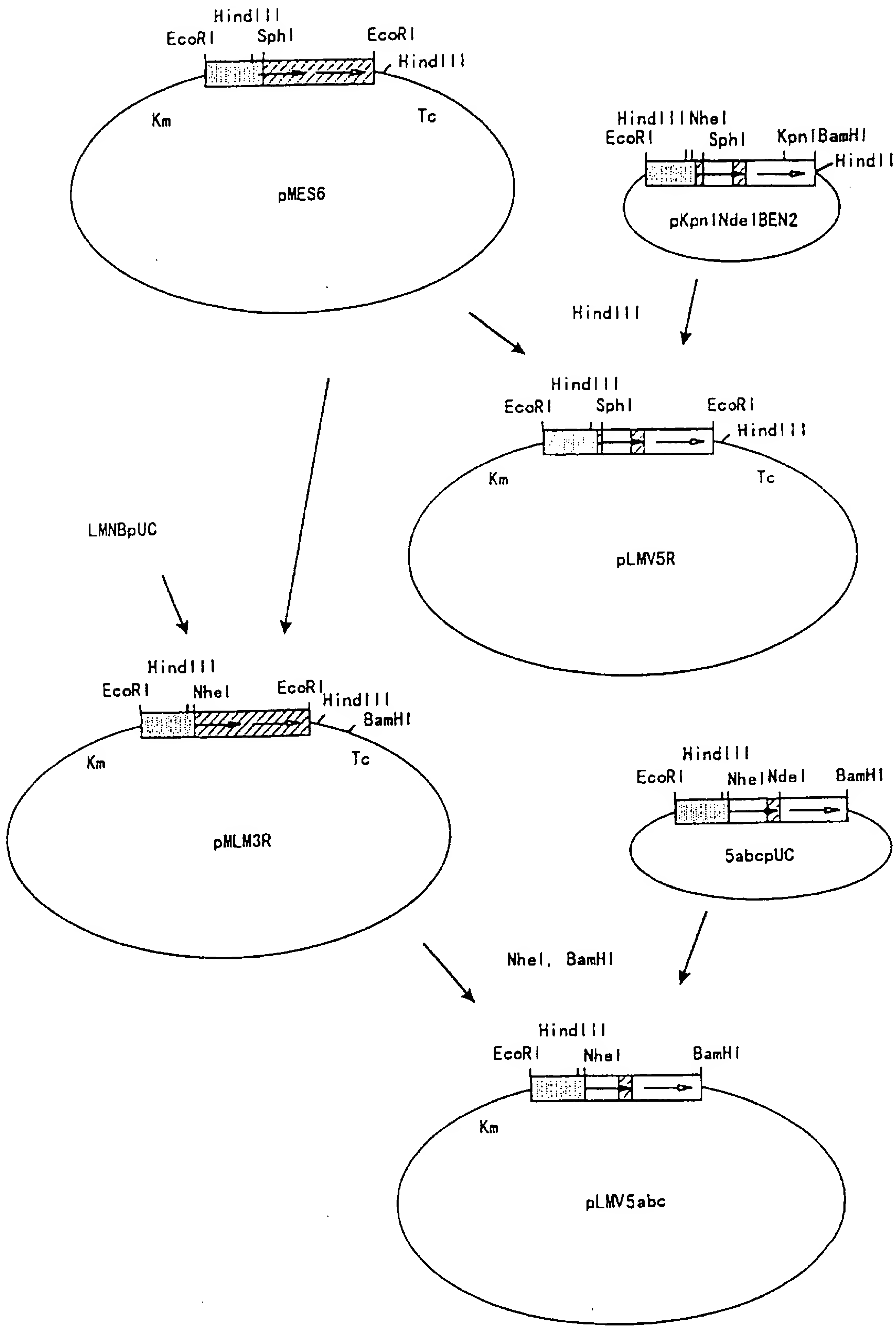

HindIII
EcoRI
SphI
EcoRI
HindIII
Km
Tc
pMES6
HindIIINheI
EcoRI
SphI
KpnIBamHI
HindIII
pKpnINdeIBEN2
HindIII
HindIII
EcoRI
SphI
EcoRI
HindIII
Km
Tc
pLMV5R
LMNBpUC
HindIII
EcoRI
NheI
EcoRI
HindIII
BamHI
Km
Tc
pMLM3R
HindIII
EcoRI
NheINdeI
BamHI
5abcpUC
NheI, BamHI
HindIII
EcoRI
NheI
BamHI
Km
pLMV5abc

Fig. 13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00345

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/20, C11D 3/386

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9425578 A1 (GISTBROCADES N.V.), 10 November 1994 (10.11.94), page 4, line 26 - line 26, See abstract. Page 10, example 1 --	1-22
X	WO 9535381 A1 (UNILEVER N.V.), 28 December 1995 (28.12.95), Page 17 table VI, page 6 line 3 5-38 --	1-22
X	WO 9530744 A2 (GISTBROCADES B.V.), 16 November 1995 (16.11.95), See claim 8 --	1-22
X	WO 9600292 A1 (UNILEVER N.V.), 4 January 1996 (04.01.96), See claim 15 --	1-22

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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"E" erlier document but published on or after the international filing date
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 November 1997	01-12- 1997

Name and mailing address of the ISA/	Authorized officer
Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Yvonne Siösteen Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00345

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9522615 A1 (NOVO NORDISK A/S), 24 August 1995 (24.08.95) -- --------	14-21

INTERNATIONAL SEARCH REPORT

Information on patent family members

01/10/97

International application No.
PCT/DK 97/00345

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		EP 0652946 A	17/05/95
		FI 945998 A	10/02/95
		JP 7508659 T	28/09/95
		PL 306812 A	18/04/95
WO 9535381 A1	28/12/95	AU 2884595 A	15/01/96
WO 9530744 A2	16/11/95	AU 2524695 A	29/11/95
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		EP 0755442 A	29/01/97
WO 9600292 A1	04/01/96	AU 2884695 A	19/01/96
WO 9522615 A1	24/08/95	AU 1806795 A	04/09/95
		CA 2183431 A	24/08/95
		EP 0746618 A	11/12/96
		FI 963266 A	21/08/96